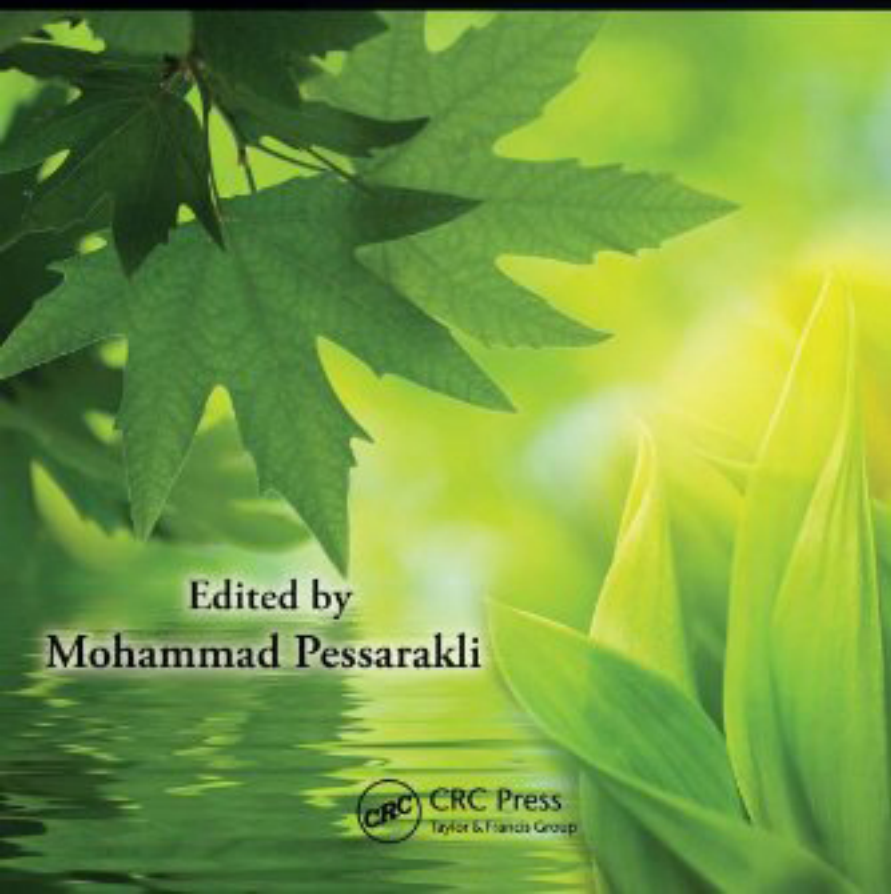


THIRD EDITION

# HANDBOOK OF PLANT AND CROP STRESS



Edited by  
**Mohammad Pessarakli**



**CRC Press**  
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THIRD EDITION

HANDBOOK OF  
PLANT AND  
CROP STRESS



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*In the memory of my beloved parents, Fatemeh and Vahab, who, regretfully, did not live to see this work and my other works, which, in no small part, resulted from their gift of many years of unconditional love.*



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# Preface

The dynamic and ever-expanding knowledge of environmental stresses, and their effects on plants and crops, has resulted in the compilation of a large volume of information since the second edition of the *Handbook of Plant and Crop Stress* was presented to scientists and professionals. This fact necessitated that this unique, comprehensive source of information be revised and all the new findings in this field be included in this updated edition. Like the first and second editions, the new edition is a unique, comprehensive, and complete collection of the issues on stress imposed on plants/crops.

More than 80% of the materials in this edition are entirely new and have been included under new titles. The remaining 20% have been updated. Therefore, almost 90% of the materials are new, thus creating what could be considered a new version.

Out of the 47 chapters, only 11 have been taken from the second edition, but have been substantially revised and updated. The other 36 chapters are entirely new.

Since the early 1900s, soil/plant scientists have observed that plant growth and crop yields decreased under salinity, drought, and/or other environmental stress conditions. Reduction in plant growth was reported as a result of a modification in the physiological process and environmental conditions that control growth. Stresses imposed on plants due to pollution or applications of agrichemicals have recently attracted the attention of scientists, investigators, and environmentalists in agriculture and related areas. The mechanisms by which salinity, drought, high/low temperatures or heat, high/low pH, high/low light, nutrient deficiency, pollution, agrichemicals, climatic changes, or any other stresses affect plant metabolism, thereby reducing plant growth and development, are still not completely understood. Among plant physiological processes, the change in nutrient uptake and metabolism induced by salt, drought, and/or other stress factors is commonly accepted by scientists as one of the most important factors responsible for abnormal plant metabolism, reduced growth, and decreased crop yield. The need for minimizing the effects of salt, drought, extreme temperatures, extreme pH, extreme light, pollution, agrichemicals, climatic changes, or any other environmental stresses on plant growth and crop yield is vital. Thus, a greater awareness of these stress factors, and problems associated with them, is essential to scientists, producers, and all involved in the field of agriculture.

This handbook is a comprehensive, up-to-date reference book that addresses issues and concerns related to plant and crop stress effectively. While there are many reference books on soil salinity, sodicity, specific plant/crop salt and water stress, pollution, and other environmental stresses, all of these exist in relative isolation, covering only one specific topic.

To solve plant and crop stress problems efficiently and effectively requires the accountability and coordination of all the factors and their interrelationship with plant/crop stress. While several authors have competently covered the many areas separately, the areas are, nonetheless, interrelated and should be covered comprehensively in a single text. Thus, the purpose of this book is to fill this niche.

The new and updated third edition of the *Handbook of Plant and Crop Stress* has been written by more than 100 contributors from 27 countries, who are among the most competent and knowledgeable scientists, specialists, and researchers in the field of agriculture. It is intended to serve as a resource for preparing for lectures as well as for conducting research. Scientists, agricultural researchers, agricultural practitioners, and students will benefit from this unique comprehensive guide, which covers plant stress problems from the soil to the atmosphere.

As with other fields, accessibility of knowledge is among the most critical factors involved with crop stress problems. Without due consideration of all the elements contributing to a specific crop stress problem, it is unlikely that a permanent solution will be achieved. Therefore, this book covers as many of the factors as possible. To further facilitate the accessibility of the desired information

in the areas of stress covered in this collection, the book has been divided into 10 parts. These include Soil Salinity and Sodicity Problems; Plant/Crop Tolerance and Stressful Conditions; Plants and Crops Responses, Physiology, Cellular and Molecular Biology, and Microbiological Aspects under Salt, Drought, Heat, Cold, Light, and Other Stressful Conditions; Plant and Crop Responses to Pollution Stress; Plant and Crop Responses to Weeds, Pests, Pathogens, and Agrichemical Stress Conditions; Genetic Factors and Plant/Crop Genomics under Stress; Examples of Empirical Investigations of Specific Plants and Crops Grown in Salt, Drought, and Other Environmental Stress Conditions; Climatic Changes, Elevated Carbon Dioxide, and Plant/Crop Responses; Future Promises: Improving Plant and Crop Adaptation/Tolerance and Cultivation under Stressful Conditions; and Beneficial Aspects of Stress. Each of these parts consists of one or more chapters so as to discuss, independently, as many aspects of the stresses as possible.

Part I consists of four chapters, including “Soil Salinity and Sodicity as Particular Plant/Crop Stress Factors”; “Soil Salinity Development, Classification, Assessment, and Management in Irrigated Agriculture”; “Soil Salinization and Management Options for Sustainable Crop Production”; and “Influence of Sodium on Soils of Humid Regions”. These chapters explain soil as a medium of crop growth, soil salinity, and sodicity problems, as well as the effects of soil salinity and sodicity on plant/crop growth.

Part II consists of seven chapters as follows: “Oxidative Stress and Antioxidative Defense Systems in Plants Growing under Abiotic Stresses”; “Antioxidant Protection during Abiotic Stresses”; “Biochemical Mechanisms for the Maintenance of Oxidative Stress under Control in Plants”; “Plant Hormone Functions in Abiotic and Biotic Stress Responses”; “Role of Proline in Plant Response to Drought and Salinity”; “Role of Dehydrins in Plant Stress Response; and Behavior of Water in Plants at Low and Ultralow Temperatures”. These chapters address plants and crop mechanisms of stress tolerance.

Part III consists of the following 12 chapters: “Germination of Seeds and Propagules under Salt Stress”; “Response of Crop Plants to Nitrogen Stress: Opportunities to Increase Nitrogen Use Efficiency”; “Photosynthesis and Light Stress in a Model Plant: Role of Chloroplast Transporters”; “Photosynthetic Pigments Apparatus in the Northern Plants”; “Modifications of the Carotenoid Metabolisms in Plastids: A Response to Stress Conditions”; “Thermoluminescence Study of Photosystem II Activity in Resurrection Plant *Haberlea rhodopensis* during Desiccation”; “Carbon Metabolism and Plant Stress”; “Protein Synthesis by Plants under Stressful Conditions”; “Heat Shock Proteins and Acquisition of Thermotolerance in Plants”; “Effect of Low Temperatures on the Structure of Plant Cells: Structural, Biochemical, and Molecular Aspects”; “Effects of UV-B Radiation on Plants: Molecular Mechanisms Involved in UV-B Responses”; and “Effect of High Temperature and UV-A Radiation on Photosystem II”. Each of these chapters provides in-depth information on each of these topics.

Part IV consists of four chapters, including “Plant Responses to Toxic Metal Stress”; “Heavy Metal Pollution: Damage and Defense Strategies in Plants”; “Heavy Metals and Plastid Metabolism”; and “Plant Responses to Cadmium and Mercury Stress”. These chapters provide detailed information on plants/crops influenced by pollution generated from either the soil, water, or the atmosphere.

Part V also consists of four chapters as follows: “Stress in Plants and Crops Induced by Parasitic Weeds”; “Involvement of Insect Pests in Plant and Crop Stress”; “Stress in Plants and Crops Induced by Herbicide-Mediated Alteration in the Population and Activity of Root-Associated Microorganisms”; and “Stress in Plants and Crops Induced by Fungal Pathogens”. These chapters discuss the interactions between weeds, pests, pathogens, and agrichemicals, and plants/crops and the potential problems caused by the application of agrichemicals to plants/crops.

Part VI consists of three chapters, including “Genetic Factors Affecting Abiotic Stress Tolerance in Crops”; “Genetic Improvement of Cold Hardiness in Bermudagrass”; and “Candidate Gene Expression Involved in Plant Drought Resistance”. These chapters present detailed and comprehensive information of all available materials on these subjects.



Several examples of empirical investigations of specific plants and crops grown under salt, drought, and/or other stress conditions are covered in Part VII, which consists of five chapters, presenting various plants and crops with different degrees of tolerance. These chapters include “Responses of Green Beans (*Phaseolus vulgaris* L.) in Terms of Dry Matter Production, Nitrogen Uptake, and Water Absorption under Salt Stress Conditions”; “Physiology and Molecular Biology of the Effects of Salinity on Rice”; “Landscaping under Water Stress Conditions”; “Turfgrass Nutrient Management under Stresses: A Part of Integrated Stress Management”; and “Nutrient Management of Golf Course Putting Greens under Stresses”.

Due to recent climatic changes and increase in CO<sub>2</sub> levels, plant resistance to these changes must be considered for cultivation under these conditions. Therefore, Part VIII, which consists of a single chapter entitled “Plant Biomass and Stem Juice of the C<sub>4</sub> Sugarcane at Elevated Growth CO<sub>2</sub> and Temperature” presents the latest information on this subject.

Part IX provides evidence and guidance on plants and crops that can be used under stressful conditions. This part consists of the following six chapters: “Improving Crop Resistance to Abiotic Stresses through Seed Invigoration”; “Physiological and Biochemical Strategies Allowing Plants/Crops to Thrive under Ionic Stress”; “Role of *Acacia ampliceps* in Managing Salt-Affected Lands”; “Adaptive Strategies of Tropical Forage Grasses to Low Phosphorus Stress: The Case of Brachiariagrasses”; “Forgotten Link in Improving Crop Salt Tolerance Research under Brackish Irrigation: Lateral Soil Salinity Gradients around Roots”; and “Improving Crop Production on Saline Soils in Arid Regions: Do We Need a Different Approach to Develop and Select Plants for These Regions?”

The important subject of beneficial aspects of stress, which has received very little attention, is covered in Part X. This part consists of a unique chapter entitled “Salinity-Induced Enhancement of Horticultural Crop Quality,” which presents available information on this subject.

Numerous figures and tables have been provided in this book to facilitate the comprehension of the materials that have been presented. An extensive index has also been generated to further increase accessibility to the desired information.

**Mohammad Pessarakli**

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# *Part I*

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## *Soil Salinity and Sodicity Problems*

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# 1 Soil Salinity and Sodicity as Particular Plant/Crop Stress Factors

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## 1.1 INTRODUCTION

Soil salinity and sodicity are among the major agricultural problems limiting plant growth and development throughout the world [2,6–8,10,12–18,22,23,26,29,30,32–35,40,42,48–52,54,59–63,66,67,71–74,80,83–86,100,105–107,109,110,112,114,115,121–124,132–134,136–144,149–152]. Salinity and sodicity problems in agriculture have an ancient history, and presently have become a very cumbersome problem in agricultural and farming activities [153]. These problems are especially of great concern for countries that their economies rely to a great extent on agriculture.

Salinity and sodicity problems are common in arid and semiarid regions, where rainfall is insufficient to leach salts and excess sodium ions out of the rhizosphere. In addition, these areas often have high evaporation rates, which can encourage increase in salt concentration at the soil surface. The arid and semiarid regions include almost one-third of the world's land [80,108,114]. According to the Food and Agricultural Organization (FAO) [46] of the United Nations, total salt-affected area of the world has been estimated to be over 800 million ha.

The presence of a cliche horizon and/or a cemented hardpan layer at varying depths plus insufficient precipitation for leaching often adds to the salt accumulation in these soils.

Newly established irrigation projects, with improper planning and management practices, may also add salts to soils [88].

Soil salinity and sodicity problems are present in nearly every irrigated area of the world and also occur on nonirrigated croplands and rangelands. Thus, virtually no land is immune to salinization. Therefore, for sustaining life on earth, control of these problems and finding new ways to utilize these extensive saline and sodic soils and water resources, at least for agricultural purposes, are vital and urgent. Reclamation, or at least minimizing the effect of salinity and/or sodicity, is important

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<sup>†</sup> Deceased.

and necessary. In this respect, proper utilization of water for both plant growth and soil salinity and sodicity control is probably of the greatest importance.

The main focus of this introductory chapter is to summarize general information on salt-affected (saline and sodic) soils, factors influencing their formation and reclamation, and discussing salinity and sodicity as plant/crop stress factors.

## 1.2 SIGNIFICANCE OF SOILS IN RESPECT OF CROP STRESS

As far as all the crops are grown on soils, soil properties have substantial influence on the life conditions of plants and crops. In nature, usually particular plant species grow on specific soils. Thus, specific relationships exist between a particular soil and the vegetation cover of that specific soil. For example, Kreeb et al. [68] investigated soil and vegetation relationships associated with sodic-saline soil surfaces.

Plant development and successful crop production require proper soil conditions, including adequate water and nutrient supply. Unfavorable soil conditions, environmental stress [2,31,130,135], salinity and/or sodicity [6–8,13,14,18,22,25,26,29,34,40,51,54,60,62,66,71,83,85,91–94, 96,97,115,124,132,133,136,137,139–142,148,149,151], and inadequate nutrient supply [98,145] have an adverse effect on the life of the plants, sometimes seriously hindering their effective production.

Based on the above facts, we can speak of stress factors originating in the soil, i.e., such unfavorable soil conditions which cause, or contribute to, the stress factors plants and crops are exposed to.

It is impossible to list all or most of such factors in a short introductory chapter. Therefore, the authors limit the range of this chapter to a general description of soil behavior and its function in nature and production as well as to an outline of one of the most serious factors originating salt-affected soils. For more in-depth information regarding salt-affected soils, the readers are referred to more comprehensive available sources [9,21,24,27,37,41,43,69,77–79,82,87,97,106,113,119,125,128,129,146,147].

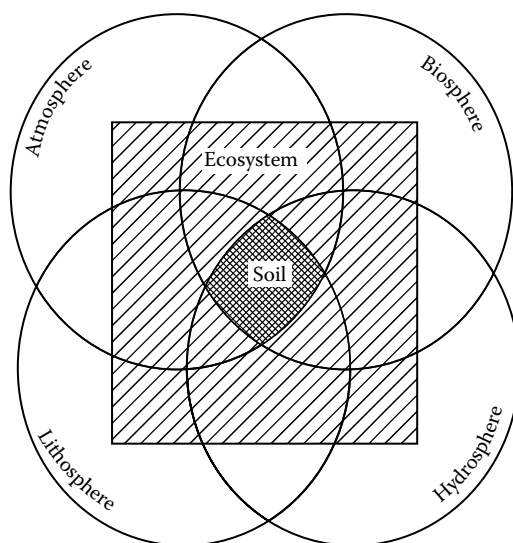
## 1.3 PLACE AND ROLE OF THE SOIL IN NATURE

It is generally accepted that the soil is a substantial part of the environment, comprising different substances and forming a special kind of ecosystem inside the given ecosystem, with various properties and attributes. It is also accepted that the soil of the continents is of high diversity, which is dealt with by several branches of soil science, e.g., taxonomy, classification, survey, mapping, etc.

The soil, or the pedosphere, which is an environmental synonym of the soils of a given territory, has a specific place in nature. It is a natural body, similar to rocks, waters, or biota, in the sense that they too have their own materials, mass and energy fluxes, development, and regularities. This fact should be mentioned because, not only in newspapers but also in technical literature, soils are frequently treated either as living substances or as nonbiological substances. Neither of these approaches is correct, because one of the characteristics of the soil is its complexity, the fact that it contains both living and nonliving substances, forming due to both biotic and abiotic processes.

The soil as a natural body is inseparable from the rocks and the crust of weathering on the surface of the continents from which it has developed, on the one hand, and from the biological processes on the other hand. The main characteristics that distinguish the soil from the rocks is the result of biological processes: the production of organic matters by the activities of microorganisms, plants, invertebrates, and other animals, and, finally, human beings, which transforms the rocks into soils, capable of supplying plants and crops with nutrients and water, and being an anchor for their establishment and stands on the land.

The processes of soil formation started concurrently with the appearance of life on the continents and continued during the billions of years of interactions between living substances and rocks under the influence of climatic conditions, with particular regard to the action of water, vegetation cover, organism (both macro and micro), geomorphological patterns, and the time factor. As a result of their interactions, specific mass and energy fluxes formed the different soil types in various environmental conditions.



**FIGURE 1.1** Schematic diagram of the interaction of lithosphere, atmosphere, biosphere, hydrosphere, ecosystems, and soils. (From Szabolcs, I., *Salt-Affected Soils*, CRC Press, Boca Raton, FL, 1989.)

With the appearance of the human race on the face of the earth, even changes in the environment became different. Due to human activities, the natural processes affected by biotic and abiotic factors accelerated, and several others that were unknown or minimal before developed.

The role of soils in nature is complex and multisided, including biospheric, hydrospheric, and lithospheric functions. Their interaction is illustrated in Figure 1.1 [128]. Figure 1.1 clearly shows that the soil is a specific body related to the ecosystem. Even the word “soil” is very often used as a synonym of ecosystem when characterizing the given ecological conditions in a certain place. If we want to be precise, we must agree that the ecosystem includes the pedon, in other words, the soils. However, the soil includes different phases (solid, liquid, gaseous), living and nonliving substances, plants, animals, microbes, and has its own energy and material fluxes. Therefore, it can be considered an ecosystem in itself. In this respect, when speaking of soils versus their plant cover, we can consider the soils of a given location as the basis, ladder, and foothold, for instance, that in savannas or in the tropical belt, a well-defined plant cover develops and very often the soil properties promote or limit the living conditions of certain plant species or associations.

Based upon the above considerations, it can be accepted that certain soil types, when discussed as the habitat for certain plant associations, are often named as the ecosystem of the plant association concerned, as the pedon includes, apart from the plants, most of the components of the ecosystem.

Evidently, the soil, as a specific natural entity, is far from being identical with the vegetation, and, in spite of their close correlation, direct conversion between soil types and vegetation is hardly possible. Still there are soil types that, more or less, determine the ecological function for certain types of vegetation either by providing beneficial conditions for their development or by limiting the ecological conditions for other types of vegetation.

This is perhaps best demonstrated in the case of salt-affected soils where high electrolyte contents of extreme pH conditions limit the development of the majority of plants and serve as a habitat only for such species that can survive or tolerate the unfavorable conditions caused by the salinity and sodicity of the soil. For example, the grass *Leptochloa fusca* that grows vigorously on the salt-affected soils can tolerate extremely saline and sodic (alkaline) conditions [69]. This species is also well adapted to the waterlogging encountered on saline and sodic (alkaline) soils. Saltgrass (*Distichlis spicata*) is another example of a highly salt-tolerant plant species that grows vigorously on saline and sodic soils [76,89–92,94–96]. In fact, the intensive investigations of the senior author

of this chapter and his coworkers on this plant species have found that this grass performed better than control when some salt was added to it during its establishment period, and so far it has been the most salt- and drought-tolerant species compared to the other highly salt-tolerant halophytes that have been tested by this investigator [89,90,94]. Other investigators [39,68,97,111] have also reported on the soil and vegetation relationships that specific plant types are adapted and growing on specific habitats. In such respects, salt-affected soils can be considered as habitat or ecosystems for halophytes, and, if we agree on this, correlations can be found between the different types of salt-affected soils and their flora and fauna as components of the ecosystem.

In order to cast light on both the theoretical and practical aspects of such considerations, it is necessary to describe briefly the properties and grouping of salt-affected soils with regard to the possibilities of the occurrence and distribution of halophytes and xerophytes developing on them.

1.4 EXTENSION AND GLOBAL DISTRIBUTION OF SALT-AFFECTED SOILS

Nearly 10% of the total land surface is covered with different types of salt-affected soils. Table 1.1 demonstrates the distribution of salt-affected soils in the world [65]. Table 1.1 shows that no continent on our globe is free from salt-affected soils. They are distributed not only in deserts and semidesert

TABLE 1.1  
Salt-Affected Soils on the Continents  
and Subcontinents

| Continent                  | Area (Millions ha) |
|----------------------------|--------------------|
| North America              | 15.7               |
| Mexico and Central America | 2.0                |
| South America              | 129.2              |
| Africa                     | 80.5               |
| South Asia                 | 87.6               |
| North and Central Asia     | 211.7              |
| South-East Asia            | 20.0               |
| Australasia                | 357.3              |
| Europe                     | 50.8               |
| Total                      | 954.8              |



FIGURE 1.2 Global distribution of the salt-affected soils.

regions, but also frequently occur in fertile alluvial plains, river valleys, and coastal areas, close to densely populated areas and irrigation systems [27,37,41,45,46,78,87,108,112,128,129].

Figure 1.2 shows the distribution of salt-affected soils throughout the world [129].

## 1.5 DEVELOPMENT AND GROUPING OF SALT-AFFECTED SOILS, PARTICULAR PLANT/CROP GROWTH STRESS FACTORS

In spite of the fact that the properties and attributes of salt-affected soils have been well known for a long time, it is appropriate to give a brief definition of this group of soils right at the start, because the salinity and sodicity (alkalinity) as well as the acidity of soils are substantial stress factors, seriously affecting the productivity of the land [2,7,8,10,12–18,22,23,25,26, 28–33,35,36,40,42, 48–52,54,56,59–63,67,70–74,80,83–87,98–100,105–107,109,110,112,114,115,121–124, 128–130,132–133,143–145,148–150,153].

Salt-affected (i.e., saline, saline-sodic, and sodic) soils usually have low biological activity both because of osmotic and ionic effects of salts and due to limitation of carbonaceous substrates. Rao and Pathak [103] reported that microbial growth was depressed in sodic (alkali) soils due to, at least in part, limitation in carbon substrate (carbon stress), and in saline soils due to salt stress.

For detailed information on the formation of salt-affected soils, the readers are referred to Szabolcs [128,129] and Pessarakli [87].

Salt-affected soils can be characterized as soils formed under the dominant influence of different salts in their solid or liquid phases, which will then have a decisive influence on the development, characteristics, physical, chemical, and biological properties, and eventually the fertility of the soil. Whenever and wherever this phenomenon occurs, it produces specific formations of soils where the high electrolyte concentration and its consequences overshadow the former soil-forming processes or former soil properties and environmental conditions, often radically changing them.

High electrolyte concentration is the only common feature of all salt-affected soils. Their chemistry, morphology, pH, and many other properties may be different, depending on the character of salinization and/or alkalization.

Salt-affected soils, in the broader sense, can be divided into the following groups:

1. Saline soils that develop under the influence of electrolytes of sodium salts with nearly neutral reaction [dominantly sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), sodium chloride ( $\text{NaCl}$ ), and seldom sodium nitrate ( $\text{NaNO}_3$ )]. These soils occur mainly in arid and semiarid regions and form a major part of all the salt-affected soils of the world.

High contents of soluble salts accumulated in these soils can significantly decrease their value and productivity.

2. Sodic (alkali) soils that develop under the influence of electrolytes capable of alkaline hydrolysis [mainly sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and sodium bicarbonate ( $\text{NaHCO}_3$ ) and seldom sodium silicate ( $\text{Na}_2\text{SiO}_3$ ) and sodium bisilicate ( $\text{NaHSiO}_3$ )]. This group is well extended in practically all the climatic regions from the humid tropics to beyond the polar circles, and their total salt content is usually lower than that of saline soils, sometimes even strongly sodic (alkaline).

Virgin sodic (alkali) soils have a high pH and high exchangeable sodium (Na) percentage (ESP) and are often barren.

Sodic soils exhibit poor physical conditions that adversely influence water and air movement in the soils. Sodicty causes soil erodibility and impairs plant growth [82,87].

3. Salt-affected soils that mostly develop owing to the presence of calcium sulfate ( $\text{CaSO}_4$ ) [gypsiferous soils] or, rarely, in the presence of calcium chloride ( $\text{CaCl}_2$ ). Gypsiferous soils can mainly be found in the arid and semiarid regions of North America, North Africa, the Near East, Middle East, and Far East, and also in Australia.

4. Salt-affected soils that develop under the influence of magnesium salts. This group occurs in arid, semiarid, and even semi-humid regions, and has a particular significance, especially those soils that have a heavy texture.
5. Acid-sulfate soils whose salt content is composed mainly of  $\text{Al}_2(\text{SO}_4)_3$  and  $\text{Fe}_2(\text{SO}_4)_3$ . This type of salt-affected soils is broadly extended in the tidal marsh areas along the seashores of all the continents. These soils are particularly common in North Europe, the western and eastern coastlines of Africa, along the coastline of South-East India, and develop on sulfurous marine sediments.

Inland acid-sulfate soils can also be found in different areas of the world, such as the western territories of the United States, Asia Minor, and China. Such soils develop as a result of fluvial glacial processes and have had no connection with seashores in recent geological times.

Evidently, the different groups of salt-affected soils have diverse physicochemical and biological properties besides the one they have in common, i.e., a comparatively high electrolyte content.

The grouping of the salt-affected soils and their properties causing plant and crop stress are presented in Table 1.2.

The five groups in Table 1.2 represent the formations of different salt-affected soils described above, indicating their chemical types, the environmental conditions where they dominate or occur, the pattern of their main adverse effect on production, and the basic methods of their reclamation. For detailed information on formation and reclamation of salt-affected soils, see Szabolcs [128,129] and Pessarakli [87].

In Table 1.2, the adverse properties of different salt-affected soils causing crop stress are also included. From these, it is clear that, in various groups, different properties are responsible for hindering the development of plants and crops by causing stress.

In saline soils, it is the high salt concentration in the solid and liquid phases that results in high osmotic pressure, hindering the normal development of plants, the stress factor is the salinity with

**TABLE 1.2**  
**Grouping of Salt-Affected Soils and Their Properties Causing Plant and Crop Stress**

| Types of Salt-Affected Soils | Electrolyte(s) Causing Salinity and/or Sodidity        | Environment                                                                                            | Properties Causing Plant and Crop Stress                          | Methods for Reclamation                                     |
|------------------------------|--------------------------------------------------------|--------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------|
| Saline                       | Sodium chloride and sulfate (in extreme cases nitrate) | Arid and semiarid                                                                                      | High osmotic pressure of soil solution, toxic effect of chlorides | Removal of excess salt (leaching)                           |
| Sodic                        | Sodium ions capable of alkaline hydrolysis             | Semiarid, semi-humid, and humid                                                                        | High (alkali) pH, poor water physical conditions                  | Lowering of neutralizing the high pH by chemical amendments |
| Magnesium                    | Magnesium ions                                         | Semiarid and semi-humid                                                                                | Toxic effect, high osmotic pressure, Ca deficiency                | Chemical amendments, leaching                               |
| Gypsiferous                  | Calcium ions (mainly $\text{CaSO}_4$ )                 | Semiarid and arid                                                                                      | Low (acidic) pH toxic effect                                      | Alkaline amendments                                         |
| Acid sulfate                 | Ferric and aluminum ions (mainly sulfates)             | Seashores and lagoons with heavy, sulfate-containing sediments, diluvial inland slopes and depressions | High acidity and the toxic effect of aluminum                     | Liming                                                      |



all its disadvantageous consequences of plant life. Apart from this, some compounds of the salt content of these soils, e.g., chlorides as toxic elements, also act as one of the stress factors.

In sodic (alkali) soils, as a rule, not the high salt concentration but the sodic (alkaline) pH value is the stress factor, particularly in cases where there is a high concentration of sodium carbonate in the solid and liquid phases of the soil. The high pH hinders the life function of crops and limits their development.

In another group of sodic (alkali) soils, which sometimes does not have very alkaline pH value (solonetz type), the comparatively low concentration of sodium salts capable of sodic (alkaline) hydrolysis constitutes a stress factor through its action, resulting in poor water physical properties in the soil. As a consequence of this phenomenon, the wilting point in the soil increases and the plants suffer from water deficiency, even in wet soils, due to the swelling of clay saturated with sodium ions ( $\text{Na}^+$ ).

In magnesium soils, which have not been adequately studied, the combination of toxic effect, calcium deficiency, and poor soil physical properties are the stress factors.

In gypsiferous soils, the acidic pH and sometimes the toxic effect of the high gypsum content, contribute to the appearance of stress factors for plant and crop life in areas with large extensions of intensively gypsiferous soils.

In acid-sulfate soils, the very high acidity, with a pH sometimes below 2, poses stress with all the adverse effects of extreme acidity. Furthermore, the high aluminum content of the soil solution has an intensive toxic effect.

Apart from this, the temporary or permanent waterlogging in such soils acts as a stress factor, hindering the normal air and nutrient regime, necessary for plant life, in these soils.

Besides the salt-affected soils developing due to natural soil-forming processes, the so-called secondary salt-affected soils have an increasing importance that is both scientific and practical. Secondary salt-affected soils are those that have been salinized due to man-made factors, mainly as a consequence of improper methods of irrigation. The extension of secondary salt-affected soils is rather sizeable, and this adverse process is as old as irrigated agriculture itself. Old civilization in Mesopotamia, China, and Pre-Columbian America fell in consequence of the salinization of irrigated land. The process is also advancing vigorously at present and more than half of all the irrigated lands in the world are under the influence of secondary salinization and/or alkalization.

When speaking of the man-made factors of salinization, we also have to mention potential salt-affected soils that are not salt-affected at present, but in case of the extension of irrigation, deforestation, overgrazing, and other man-made measures, can and will be salinized unless the necessary preventive procedures are undertaken in due time. No global records are available of the size of potential salt-affected soils; however, the area that they cover is larger than that of existing salt-affected soils.

Secondary salt-affected soils can be divided into the following two categories:

1. Secondary formation of salt-affected soils caused by irrigation.

In spite of the negative experiences, the salinization of irrigated and surrounding areas has not diminished. On the contrary, it is still on the increase.

According to the estimates of the FAO and UNESCO (the United Nations' Educational, Scientific and Cultural Organization), as much as half of all the existing irrigation systems of the world are, more or less, under the influence of secondary salinization, alkalization, and waterlogging. This phenomenon is very common not only in old irrigation systems but also in areas where irrigation has only recently begun.

According to the estimates of the above mentioned agencies, 10 million ha of irrigated land are abandoned yearly because of the adverse effects of salinity due to irrigation, mainly secondary salinization and alkalization.

The mentioned losses and damages are not evenly distributed among the irrigating countries. In some of them, the damage may be relatively small, while in others it actually constitutes the major problem in agriculture or even in the national economy of the country in question. In this respect, unfortunately, there are countless sad examples.

In Pakistan, Ahmad [4] carried out statistical analyses in respect of secondary salinized land. According to his data, out of 35 million ac (approximately 16 million ha) of total irrigated territory, salinized areas account for 5.3 million ac (approximately 2.4 million ha) after a few years of irrigation. He indicated among the causes of secondary salinization in Pakistan the joint effect of irrigation and ground water. According to Zavaleta [147], practically all irrigated alluvial soils in Peru show the features of salinity and sodicity (alkalinity). It is known from FAO reports [45,46] and the papers of Kovda [64] that more than 40% of irrigated soils in Iraq and Iran are affected by secondary salinization. A country report by FAO [45] on salinity in Syria estimated the adverse effects of salinity as follows:

- a. In more than 20,000 ha, salinity developed to a level where these soils had to be taken out of cultivation, and the loss is estimated at a total of 30,000 ton of cotton per year.
- b. In about 30,000 ha, the yield decreased by 50% and the total loss is estimated at 20,000 ton of cotton per year.
- c. In about 60,000 ha, the yield decreased by 20%, and the total loss is estimated at about 18,000 ton of cotton per year.

At present, no continent is free from the occurrence of this very serious phenomenon. In Argentina, 50% of the 40,000 ha of land irrigated in the nineteenth century are now salinized. In Australia, secondary salinization and alkalization take place in the valley of the river Murray, and in Northern Victoria 80,000 ha have been affected. The same phenomena can be observed in Alberta, Canada, and similar processes have been recorded in the northern states of the United States, where irrigation was introduced much later than in the dry west. It is noteworthy that these last examples, and many other irrigated regions, are far from being arid areas and the majority of salt accumulations are associated with the sodium salts capable of sodic (alkaline) hydrolysis, and not with the neutral sodium salts that we are familiar with in desert and semidesert areas.

The more recent reports of the FAO [46] of the United Nations estimated the salt-affected areas due to irrigation in the developing countries, including the above mentioned ones (Iran, Iraq, Pakistan, and Syria) much higher than the previous reports.

2. Secondary formation of salt-affected soils caused by human activities other than irrigation. When speaking of secondary salinization, most people think of irrigation and drainage. However, there are also other anthropogenic factors causing this adverse phenomenon. It is true that the majority of secondary salt-affected soils develop as a result of improper methods of irrigation, but there are other human effects that more and more often trigger this process in many places, both in arid and humid areas.

Some of these anthropogenic processes are, including but not limited to, the following:

- a. Overgrazing

This process occurs mainly in arid and semiarid regions, where the natural soil cover is poor and scarcely satisfies the fodder requirement of rather extensive animal husbandry. If, due to overgrazing, the natural vegetation is sparse or annihilated, progressive salinization develops and, step by step, the scarcity of the plant cover becomes increasingly pronounced. Sometimes, the process ends in desertification because even the poor pasture diminishes and no other fodder resources are available. According to Theunissen [131], the gradual decline in the ecological condition of natural pastures as a result of overgrazing and the application of insufficient management decisions, coupled with the detrimental effects of long-term drought, has left extensive areas of high potential grazing land in southern Africa in urgent need of restoration. However, due to the limited number of grasses currently available for rehabilitating and restoring the vast number of different habitats encountered, selecting indigenous grasses suitable for restoration of denuded areas in the arid and semiarid grasslands of Southern Africa was initiated.

b. Deforestation in semi-humid and semiarid areas

Particularly, in the last few decades, it has become evident that deforestation results in many tropical and subtropical countries in the salinization and alkalization of soils due to the effects of soil migration both in the upper and the lower layers. In South East India, e.g., vast territories of former forest land became intensely saline and sodic (alkaline) in a few years after the annihilation of the woods. Similar phenomena occurred in the forest steppe areas in Russia, Iran, East-Central Europe, and Latin America.

c. Salinization caused by contamination with chemicals

In spite of the fact that the amount of chemicals applied in agriculture is practically negligible, in comparison to the salt content of several soils, we have considered the fact that this kind of salinization more and more often occurs in modern intensive agricultural production, particularly in greenhouses and intensive farming systems. When production takes place in semi-closed systems (e.g., greenhouses), where the chemicals applied will not be removed regularly, the accumulation of salts or their components becomes possible in the upper layer of the soil, resulting in salinity and sodicity (alkalinity). In Japan, the Netherlands, and other countries with intensive agriculture, and particularly horticulture, such type of salinization more and more frequently appears, causing serious losses of crop yields.

d. The accumulation of airborne or waterborne salts

Due to the concentration of industrial plants, the emission of chemical compounds may accumulate in the soil and, if their concentration is high enough, they result in salt accumulation in the upper layer of the soil.

A similar phenomenon appears when, due to water system regulations, sludge water disposal, and other hydrotechnical measures, water with considerable salt concentration contaminates the upper soil layer, causing salinization and/or alkalization.

## 1.6 RECLAMATION OF SALT-AFFECTED SOILS, RELIEVE OR ELIMINATION OF PARTICULAR PLANT/CROP STRESS FACTORS

The major environmental stresses caused by soil salinity and sodicity have existed long before the agricultural practices have started. Soil salinity and sodicity have a substantial effect in reducing agricultural production worldwide [2,6–8,10,12–20,22,23,26,29,30,32–35,40,42,48–52,54,59–63,66,67,71–73,80,83–86,100,105,107,109,110,112,114,115,121–124,132–134,136–144,148–150,152]. This has a major impact on increased food and feed insecurity globally, particularly in developing countries that are more prone and vulnerable to salinization and desertification due to lack of advanced technology, adequate education, and other socioeconomical and technological problems. Population growth and increasing demand for food and agricultural products necessitate using the salt-affected soils and marginal lands for food production. These soils are needed for the agricultural extension and, hence, reclamation is required. Reclamation is needed on the millions of hectares of slowly permeable salt-affected (i.e., saline–sodic and sodic) soils throughout the world [2,46,55,63,87,112,124,150].

Different techniques of reclamation and preventive measures or management practices are used for reclamation of salt-affected soils and reducing the salt contents of the growth medium or to find more stress-tolerant plant/crop species and cultivars via genetic engineering to combat salinity stress. These management practices were aimed to enable plants to grow in saline and sodic conditions to utilize salt-affected soils for agricultural practices and food production [2,3,5,10,12,15–20,23,28,30,32,33,35,38,42,44,47–49,52,56,59,61,63,69,72,80,81,84,86,87,99,100,105,107–110,114,117–119,121–124,126,131,134,138,143,144,149,150,152]. Saline soils are usually reclaimed by leaching the salts out of the soil through irrigation and drainage systems, whereas reclamation of sodic (alkaline) soils requires application of chemical amendments followed by the leaching process.

Present recommendations for reclamation of the salt-affected soils are usually based only on relatively simple and often empirical relations. Various amendments and management strategies have been used for reclamation of the salt-affected soils. To evaluate particular reclamation strategies, some specific considerations should be noted as follows:

1. The quantity of water needed
2. The quality of water needed
3. The quantity of amendments to be used
4. The type(s) of amendment(s) to be used
5. The time required for reclamation to be completed

Chemical reactions such as cation exchange, precipitation, and dissolution of solid phases (reclamation amendments) and the soil hydraulic properties and corresponding changes in the water flow and solute transport rates must be considered [119].

Among the various reclamation practices, usually, a combination of added gypsum amendment and crop rotation has been proven the best.

Reclamations of salt-affected (saline-sodic and sodic) soils by chemical amendments has become cost-intensive and requires high capital investment, and are not always a practical solution to the problem of soil salinity and sodicity. Therefore, biotic approach such as cultivation of salinity- and sodicity-tolerant plants and crops on salt-affected soils, i.e., “saline agriculture,” may be another alternative.

Cultivation of different salinity- and sodicity-tolerant plant types and species have been used by several investigators, i.e., grasses [44,62,69,75,76,89–91,93–97,99,131,140,148], agronomic crops [5,15,16,19,20,23,42,47,48,61,63,72,73,81,86,108,114,121–123,149,150], forest species, and trees [10,28,54,117,118,126] for reclamation purposes. These plants can mobilize the native lime (calcium carbonate,  $\text{CaCO}_3$ ) in these soils through root action, a substitute for the chemical approach. Qadir et al. [97], studying the combination of chemical amendments and biological (using plants) reclamation technique, reported that the soil treated with gypsum at a high rate (100% GR, grade reagent) removed the greatest amount of  $\text{Na}^+$  from the soil columns and resulted in a marked decrease in soil salinity (EC, electrical conductivity) and sodicity, sodium absorption ratio (SAR), and ESP (exchange sodium percentage). The performance of grass treatment in enhancing the leaching of  $\text{Na}^+$  was between the gypsum treatments.

According to Kumar [69] and Qadir [97], the grass, *Leptochloa fusca*, was very useful and effective in the reclamation of salt-affected soils. This plant can tolerate extremely saline and sodic (alkaline) conditions. Since its growth is not affected by gypsum application, planting with *Leptochloa* is an alternative biological rather than a chemical method for the reclamation of sodic (alkaline) soils. This plant is also well adapted to the waterlogging encountered on saline and sodic (alkaline) soils. The plant improves the soil's physical, chemical, and biological properties so that within 2 or 3 years many commercial and forage crops can be grown on the soil [69]. *Leptochloa* excretes salts through specialized glands and is, therefore, reasonably palatable to farm animals. It must be noted that because of its vigorous growth on sodic (alkaline) soils, *Leptochloa* does not allow satisfactory growth of companion plant species, especially in the initial years of soil reclamation.

Subramaniam and Babu [126] also used a forest shrub species for reclamation of sodic soils. According to these investigators [126], *Sophora mollis*, which is a shrub to medium-sized tree and is used for both fodder and firewood, can be used in the reclamation of sodic (alkaline) soils.

Kilic et al. [63] investigated the salt-removing capacity of purslane (*Portulaca oleracea* L.) by studying different stress criteria and by tracking its salt removal from germination to harvest. The results of their study showed that purslane could cumulatively remove considerable amounts of salt from the soil if practical to cultivate as an intercrop all year round.

Saltgrass (*Distichlis spicata*) that has been found the only vegetation cover on a highly sodic (alkaline) soil in Wilcox Playa, Arizona [92] can also be very effective in reclamation of saline

and sodic soils. As mentioned earlier, the senior author of this chapter and his coworkers found this grass to be very high salt-tolerant plant species that grows vigorously on saline and sodic soils [76,89–92,94–96]. Compared to the other highly salt-tolerant halophytes that have been tested by this investigator [89,90,94], so far, this grass has proven the most salt and drought tolerant of all the tested species.

Although slow, definite improvement is achieved in the physicochemical properties of the salt-affected soils by encouraging the vegetation growth on such lands. The tree species in general are effective in improving the soil properties as reflected by the changes in physicochemical characteristics of the soil such as bulk density (BD), water holding capacity (WHC), hydraulic conductivity (HC) and pH, EC, OC (organic carbon), N (nitrogen) and exchangeable cations ( $\text{Na}^+$  and  $\text{Ca}^{++}$ ) [117].

Due to the low biological activity and depressed microbial growth of salt-affected (i.e., saline, saline–sodic, and sodic) soils, there is a need for applying organic amendments (i.e., plant residue or manure) during sodic (alkali) soil reclamation. In reclamation of saline soils, organic amendments must be applied following the leaching process.

Kumar et al. [70] conducted a combination of biological and chemical reclamation study on a highly sodic (alkaline) soil. These investigators [70] found that rice produced satisfactory yields in the first year of gypsum application, but sorghum and *Sesbania* yields were very poor. The yield of *Leptochloa* was not affected by gypsum application. In their crop rotation practice, Kumar et al. [70] reported that the green forage yield of sorghum was greatest when sorghum followed *Leptochloa* grown for 2 years and the harvested grass was left to be decomposed on the site.

In a biological reclamation study of saline soils, Helalia et al. [53] reported that amshot grass significantly reduced the soil salinity compared to either ponding or gypsum application, and this grass produced a higher fresh yield than clover cultivated in such soils.

The above findings indicate that biological reclamation with the salinity- or sodicty-tolerant plants (i.e., *Leptochloa*, grasses, shrubs, or trees) is a proper substitute for chemical reclamation with gypsum, and the former has an economic advantage over the latter.

Yildirim et al. [144] evaluated the effects of selected biological treatment on direct seeded and transplanted squash plant growth and mineral contents under salinity stress. These investigators reported that salinity negatively affected growth of squash; however, biological treatments significantly increased fresh weight compared to nontreated plants that were under salt stress. They also found biological treatments increased the uptake of potassium compared to the nontreated control in both direct seeded and transplanted squash. Based on their results, these investigators concluded that alteration of mineral uptake may be one mechanism for the alleviation of salt stress, and the use of biological treatments may provide a means of facilitating plant growth under salt stress conditions.

Compost or any other organic materials is recommended to be used during the reclamation process of the salt-affected soils. The results of a field experiment conducted by Avnimelech et al. [21] verified that compost application improved both physical and chemical conditions of saline and sodic (alkaline) soils. Compost application to such soils is expected to release acids, which would ultimately lead to the replacement of exchangeable sodium by calcium. In addition, compost application would stabilize soil structure and enhance plant growth. These investigators [21] found that the municipal solid waste compost application was equivalent or even superior to the addition of gypsum, the most common amendment used to reclaim sodic (alkaline) soils. This was evident from the substantial increase in crop yields. The combined application of compost and gypsum raised yields to the levels equal to that of the commercial fields.

In a field experiment, Batra et al. [24] compared the microbiological and chemical amelioration of a highly deteriorated sodic (alkaline) soil using two reclamation technologies:

1. Growing Karnal grass (*Leptochloa fusca*) as a first crop with no chemical amendment (biological reclamation)
2. Gypsum application as a chemical amendment for different crop rotations

These investigators [24] reported that the microbiological properties changed more than the chemical properties of sodic (alkali) soil as the time period advanced.

In a biological reclamation study carried out on saline soils, Apte and Thomas [11] found that a brackish water, nitrogen-fixing cyanobacterium, *Anabaena torulosa*, could successfully grow and fix nitrogen on moderately saline soils (EC of 5–8.5 dS m<sup>-1</sup>). These investigators [11] reported that cyanobacterium exhibited high rates of nitrogen fixation and substantially enriched the nitrogen status of saline soils. However, permanent removal of Na<sup>+</sup> from saline soils using cyanobacteria or any other microorganisms may not be possible, since Na<sup>+</sup> is released back into the soil subsequent to the death and decay of cyanobacteria or other microorganisms. Amelioration of soil salinity by simultaneous application of *Anabaena torulosa* during crop growth seems to be an attractive possibility for reclamation, especially since it can also supplement the nitrogen requirement of the crops growing on these soils.

Blue-green algae that tolerate excess Na and grow extensively on the soil surface in wet seasons were found effective in sodic soils reclamation [102]. However, a permanent reclamation of such soils by using only blue-green algae as a biological amendment to achieve sodic (alkali) soil reclamation is neither possible nor comparable with an effective chemical amendment such as gypsum.

In the reclamation process of the saline soils, de Villiers et al. [39] compared different annual and perennial species. Of the six species tested, the perennials seemed to be more effective and better suited for rehabilitation purposes under saline soil conditions.

The type of chemical compound being used also influences the reclamation process of salt-affected soils. Sharma and Upadhyay [116] reported that, among the up-to-date known chemical compounds, cyclohexathiazonium chloride (S-6N-4)-2+Cl<sup>-</sup>2 is the best and the most suitable chemical to reclaim the sodic (alkaline) soil at any pH of the soil.

When good quality water is not available for leaching the salts out of the soil, low-quality water can be used for the initial stages of reclamation. In this regard, Singh and Bajwa [120] studied the effects of gypsum and sodic irrigation on the precipitations of Ca<sup>++</sup> and removal of Na<sup>+</sup> from a sodic soil reclaimed with different levels of gypsum and growth of rice in a greenhouse experiment. Dubey and Mondal [43] also used low-quality saline water in conjunction with organic and inorganic amendments for initial stages of reclamation of sodic soils. Using low-quality water, Joshi and Dhir [58] evaluated the rehabilitation of degraded sodic soils using residual sodium carbonate water (low-quality water) combined with gypsum treatment and found that the combination treatment was effective in lowering the soil SAR (sodium absorption ratio) and improved water infiltration rate. In the first year of gypsum treatment, it was possible to establish the crop. In the second year, moderate productions of wheat (2610 kg ha<sup>-1</sup>) and raga (*Brassica* sp.) (2000 kg ha<sup>-1</sup>) were obtained [58].

Using the most common technique, irrigation water and drainage system, for reclamation of the salt-affected soils, the results of an investigation carried out by Millette et al. [77] demonstrated the ability of fall irrigation to leach salts from the surface soil during a period of low consumptive use, which could lead to reclamation. Long-term monitoring would be required to determine whether a further and permanent decline in salinity could be achieved.

Concerning other reclamation materials and techniques, results of Jones et al. [57] indicate that acid whey is effective in reclaiming sodic soil by lowering ESP, SAR, and pH and by improving infiltration rate. Rao and Leeds Harrison [104] used simulation models for desalinization of a drained two-layered saline soil using surface irrigation for different water management practices to increase leaching efficiency. Based on image elements and their correlation with the ground features, Rao et al. [101] suggested categorizing sodic soils in moderately and strongly sodic groups. The delineation thus made would help the execution of a reclamation program for sodic soils at the study sites. Abdel-Hamid et al. [1] monitored soil salinity in the northern Nile delta Egypt by using data collected via landsat and geographic information system (GIS). The collected data were used in making recommendations for reclamation of the saline soils of the Nile delta area.

The vast area of salt-affected soils still remains a burden for the societies, particularly the undeveloped countries, in need of adequate resources to reclaim them with the available technology

involving initial heavy investments. The process of degradation, which has been due to reckless destruction of vegetation, can be reversed by reestablishment of vegetative cover, which results in slow but definite improvement in such soils. This phenomenon has been demonstrated a great deal by various parameters influencing the soil welfare in several investigations which show a positive sign of improvement both in terms of physical and chemical properties of the salt-affected soils. Such soils should, therefore, be brought under any type of vegetation (i.e., sod, shrub, tree) cover, if not found economical for regular farming and growing agronomic crops, and taken care by the community for posterity [117].

Even by the execution of the reclamation processes, nutrient status and their behavior in salt-affected soils (i.e., saline-sodic and sodic soils) during reclamation by crop rotation and chemical amendments requires a comprehensive assessment. This is because, usually, during the leaching process of the soluble salts and the exchangeable sodium, some soil nutrients are also lost and leached out of the soil. In this regard, several investigators [28,36,98,127,145] have studied nutrient status and behavior during the reclamation processes. Swarup et al. [127] reported the effect of gypsum on the behavior of soil phosphorus during the reclamation of a sodic soil. According to Bhojvaid et al. [28], soil nutrient status under the tree plantation was higher than that of the non-sodic farm soil. This finding confirms that successful tree plantation may restore the productivity and fertility of highly degraded sodic soils.

Regardless, the techniques used in reclamation of salt-affected soils, post-reclamation management practices, i.e., proper choice of crops, crop rotation, method of irrigation, quality and quantity of water used for irrigation and reclamation, fertilization, and the economics of reclamation must be taken into consideration and followed to achieve successful results.

## 1.7 CONCLUDING REMARKS

In this chapter, information has been given on the important functions of the soil in relation to soil-originated stress factors for plant and crop growth and development as well as a little more detailed information of particular problems related to salt-affected soils, their formation and reclamation.

The properties of the stress factors for plant and crop growth originating in soil are diverse and multisided. We know comparatively little about the up-to-date orientation and, particularly, for finding methods to improve the situation and ensure better plant and crop growth and development. Therefore, target-oriented studies of the different kinds of soil-originated stress factors for plant and crop growth and development are necessary so that the complex correlations and actions in the soil-plant-water system can be disclosed with the purpose of a better characterization of stress factors on the one hand, and improving the environmental and production conditions on the other hand.

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# 2 Soil Salinity Development, Classification, Assessment, and Management in Irrigated Agriculture

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## 2.1 INTRODUCTION

Arid zones receive inadequate and irregular precipitation to accomplish leaching of salts originally present in the soil profile. Normally when the precipitation is more than 1000 mm per annum, salinity should not develop. This is not the case in arid zones; therefore, salts accumulate in soils. Salt buildup in concentrations detrimental to plant growth is a constant threat in irrigated crop production.

In arid and semiarid regions, evapotranspiration is higher than the total annual rainfall. Therefore, rainfall contributes insignificantly to groundwater recharge, and hence there is a general shortage of fresh quality water to offset the total agriculture water demand in these countries. The shortage of fresh water necessitates the use of marginal quality ground water, such as brackish and saline, for irrigated agriculture. This is highly demanded in water-scarce regions. The improper use of saline/brackish water in irrigated agriculture often introduces salinity and sodicity problems and the soil if not properly managed can reach a condition where it cannot be exploited to its full production capacity. Under such conditions, irrigated agriculture has faced the challenge of sustaining its productivity for centuries, particularly soil and water salinity, poor irrigation, and drainage management continue to plague agriculture especially in arid and semiarid regions (Tanji, 1996).

If soil becomes saline and sodic, it creates plant- and soil-related problems that restrict plant growth through undermining soil quality, and hence many plants either fail to grow in saline soils or their growth is retarded significantly; however, few plants grow well on saline soils (Maas, 1990). Therefore, soil salinity often restricts options for cropping in a given area. Because of this reason, understanding salinity in irrigated agriculture fields is essential for their precise management. Salinity management is highly site specific and depends on factors such as site characteristics, nature of soils, and local hydrological conditions. Soil salinity and sodicity are global issues and not restricted to one country or region.

Once the soil salinity and sodicity are diagnosed and site characteristics are established, integrated management and reclamation strategies specific to the site can be formulated for better results and long-time sustainability of irrigated agriculture.

### 2.1.1 SALINITY AND SODICITY: A GLOBAL SCALE PROBLEM

Planet Earth consists of land surface of about  $13.2 \times 10^9$  ha, out of which only  $7 \times 10^9$  ha are arable and only  $1.5 \times 10^9$  ha are cultivated (Massoud, 1981). Of the cultivated lands, about  $0.34 \times 10^9$  ha (23%) are saline and another  $0.56 \times 10^9$  (37%) are sodic. Older estimates (Szabolcs, 1989) suggest 10% of the total arable land to be affected by salinity and sodicity, and extends over more than 100 countries and almost all continents.

### 2.1.2 SALINITY AND SODICITY

Some people get confused between salinity and sodicity. *Salinity* is a measure of the concentration of all the soluble salts in soil or water. It is expressed as decisiemens per meter ( $\text{dS m}^{-1}$ ) or millisiemens per centimeter ( $\text{mS cm}^{-1}$ ). If we want to keep our soils productive, we need to identify potential salinity problems and be ready with remedies or actions to help reduce the effects or avoid them in the first place. *Sodicity* is a measure of sodium ions in soil or water relative to calcium and magnesium ions (Richards, 1954). It is expressed either as the sodium adsorption ratio (SAR) or as

the exchangeable sodium percentage (ESP). If the SAR of the soil equals or is greater than 13 or ESP equals or is greater than 15, the soil is termed sodic (Richards, 1954).

### 2.1.3 CAUSES OF SALINITY DEVELOPMENT

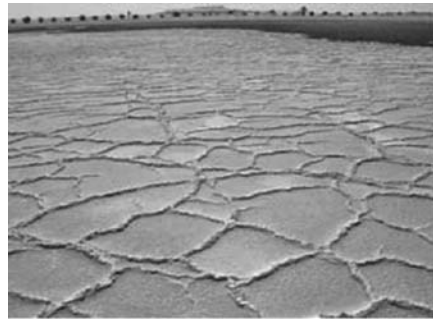
There may be a number of causes (Plate 2.1) of soil salinity development: (1) inherent soil salinity (parent material); (2) seawater intrusion to coastal areas; (3) uses of brackish/saline water in farming and urban landscapes area; (4) restricted drainage developed into a high water table; (5) low rainfall; and (6) high rate of evapotranspiration. The rainfall contributes 10–200 kg salts per year per ha, depending on the vicinity of the area to the sea or inland.

In farming areas, the continuous pumping of ground water and subsequent use for irrigation purposes (recycling) usually lowers the water table; however, this practice resulted in an increase in water salinity and covert normal soils to become saline with low productivity. These soils need attention for their management and reclamation.

Pumping groundwater to alleviate surface salinity and to lower water table is an effective way, with the condition that ground brackish water is not used directly for irrigation, but with some management, e.g., conjunctive or cyclic use. In areas where water table is high and persistent, the imbalance in the natural water, the clearing of vegetation and the general absence of deep rooted trees, and the absence of adequate drainage cause soil salinity. As the groundwater rises, it brings salt to the surface through capillary rise and subsequent evaporation, which can be harmful to plants by reducing yields. The quality of the groundwater used for irrigation and its rates of recharge are critical too. These considerations make land-water management in irrigated arid lands a delicate task. Subject to these limitations, irrigation helps reduce risks linked to soil moisture stress and enhances yield.



(a)



(b)



(c)



(d)

**PLATE 2.1** Soil salinization, waterlogging, and plant growth. (a) Patchy salinity in wheat field. (b) Salt accumulation through sea water intrusion—salt flat. (c) Affect of salinity on date palm trees. (d) Waterlogging in forestry field.



The groundwater usually rises 0.6–1.5 m or more in the soil above the water table by capillarity, depending upon texture, structure, and other factors. The water reaching the surface evaporates, leaving a salt-deposit typical of saline soils. Generally, water table below 2 m is considered safe for irrigated agriculture.

### 2.1.3.1 Salinity Development: Hypothetical Cycle

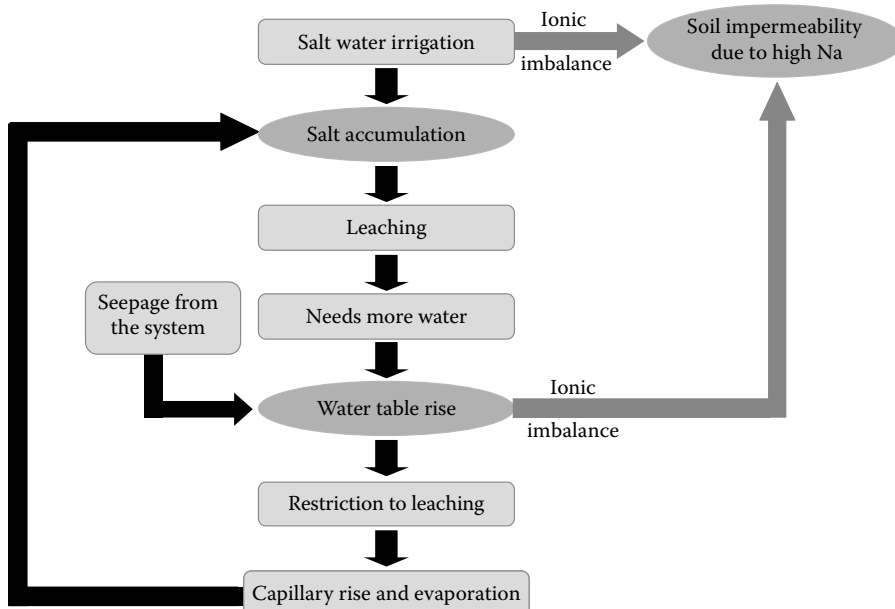
Recently, Shahid et al. (2010) have published a hypothetical salinization development cycle in irrigated agriculture fields (secondary salinization). Figure 2.1 depicts poor irrigation and drainage management and high temperature as the main causes of secondary salinization.

### 2.1.3.2 Dryland Salinity Development

In Australia, dryland salinity is very common and is developed due to clearing of trees to convert the area to arable agriculture. In the former, the rainfall is compensated through high evapotranspiration with no or insignificant leaching; in the latter case, low evapotranspiration relative to former lead the extra rain to leach down and with poor drainage condition, water table developed and subsequent evaporation caused dryland soil salinization.

## 2.1.4 DAMAGE DUE TO SALINITY

An exact estimate of losses caused due to salinity in an area is difficult to know; however, it is clear that losses may be quite considerable, and high cost of work to be done to control salinity must also be added. Different types of salinity damage are; saline water tables can cause productive land to become barren; soil salinity also enhances “erosion” and loss of farm income; salinity can deteriorate the quality of drinking water; in salt-affected areas, roads and building foundations are weakened by high salty water tables and high water table also affects biological activity in the soil.



**FIGURE 2.1** Hypothetical salinization cycle. (From Shahid, S.A. et al., Mapping and monitoring of soil salinization, remote sensing, GIS, modeling, electromagnetic induction and conventional methods—case studies, in *Proceedings of International Conference on Management of Soil and Groundwater Salinization in Arid Regions*, Sultan Qaboos University, Sultanate of Oman, January 11–14, 2010. Volume 1: Keynote papers and Abstracts, pp. 59–97.)

2.1.5 QUICK FACTS ABOUT SALINITY AND PLANT GROWTH

Proper plant selection is one way to moderate yield reductions caused by excessive soil salinity. The stage of plant growth has a direct bearing on salt tolerance. Generally, the more mature the plant, the more tolerant it is to salt. Most fruit trees are more sensitive to salt than are vegetable, field, and forage crops and generally, vegetable crops are more sensitive to salt than are field and forage crops.

2.1.6 INDICATORS OF SOIL SALINIZATION

Once soil salinity is developed in irrigated agriculture fields, it starts showing its effects on soil properties and plant growth. The white salt crust, reduced plant vigor, salt stain on dry soil surface, affected area worsen after rainfall, marked changes in leaf color and shape, and presence of naturally growing halophytes and trees are either dead or dying are the indicators of soil salinity, which can be observed in the field without laboratory analyses.

2.1.7 CLASSES OF SOIL SALINITY AND PLANT GROWTH

Electrical conductivity of the soil saturation extract (ECe) is the standard measure of salinity. Richards (1954) has described general relationship of ECe and plant growth.

| Class                  | ECe (dS m <sup>-1</sup> ) | Plant Growth                                           |
|------------------------|---------------------------|--------------------------------------------------------|
| 0 Nonsaline            | 0–2                       | Salinity effects mostly negligible                     |
| 1 Very slightly saline | 2–4                       | Yields of very sensitive crops may be restricted       |
| 2 Slightly saline      | 4–8                       | Yields of many crops restricted                        |
| 3 Moderately saline    | 8–16                      | Only tolerant crops yield satisfactory                 |
| 4 Strongly saline      | >16                       | Only a few very salt-tolerant crops yield satisfactory |

2.2 CLASSIFICATION OF SALT-AFFECTED SOILS

A soil, which contains sufficient soluble salts in the root-zone to impair the growth of crop plants, is defined as “saline.” However, because salt injury depends on species, variety, growth stage, environmental factors, and nature of the salts, it is very difficult to define a saline soil precisely. The definitions are based on salt content either alone or in conjunction with texture, morphology, or hydrology (Richards, 1954; Northcote and Skene, 1972; FAO-UNESCO, 1974; Soil Science Society of America, 1978). The most widely accepted definition of a saline soil is one that gives an electrical conductivity of extract from saturated soil paste (ECe exceeding 4 dS m<sup>-1</sup> at 25°C), while FAO-UNESCO (1974) mapped soils with ECe exceeding 15 dS m<sup>-1</sup> as strongly saline soils or solonchaks.

2.2.1 U.S. SALINITY LABORATORY STAFF (RICHARDS, 1954) CLASSIFICATION

The term “salt-affected” soil is being used more commonly to include saline, saline-sodic, and sodic soils, which are clearly differentiated by Richards (1954). The term “alkali” to describe soils with excess exchangeable sodium (ES) is being discouraged due to its ambiguity (Overstreet et al., 1951).

*Saline* soils are those which have pHs usually less than 8.5, ECe > 4 dS m<sup>-1</sup> and ESP < 15. The high ECe with low ESP tends to flocculate soil particles into aggregates. The soils are usually recognized by the presence of white salt-crust during some part of the year. Permeability is either greater or equal to those of similar normal soils.

*Saline-sodic* soils contain sufficient soluble salts (ECe > 4 dS m<sup>-1</sup>) to interfere with the growth of most crop plants and sufficient ESP (>15) to affect the soil properties and plant growth adversely by the degradation of soil structure. The pHs may be less or more than 8.5.

*Sodic* soils contain  $\text{ESP} > 15$  and  $\text{ECe} < 4 \text{ dS m}^{-1}$  and pHs generally range between 8.5 and 10 and may even be as high as 11. The low  $\text{ECe}$  and high  $\text{ESP}$  tends to deflocculate soil aggregates and hence lower their permeability.

### 2.2.2 FAO-UNESCO CLASSIFICATION (1974)

Salt-affected soils (halomorphic soils) are also indicated on the soil map of the world (1:5,000,000) by FAO-UNESCO (1974) as solonchaks and solonetz. The origin of both solonchak and solonetz are Russian. Solonchaks are soils with high salinity ( $\text{ECe} > 15 \text{ dS m}^{-1}$ ) within 125 cm of the soil surface. The FAO-UNESCO (1974) divided solonchaks into four mapping units: (1) *orthic solonchaks*—the most common solonchaks; (2) *gleyic solonchaks*—with groundwater influencing the upper 50 cm; (3) *takyric solonchaks*—solonchaks in cracking clay soils and; (4) *mollic solonchaks*—solonchaks with dark colored surface layer, often high in organic matter. Soils with a lower salinity than solonchaks, but higher than  $4 \text{ dS m}^{-1}$  are mapped as “saline phase” of other soil units.

A “solonetz” is a sodium-rich soil that has an  $\text{ESP} > 15$ . The solonetz are subdivided into three mapping units: (1) *orthic solonetz*—the most common solonetz; (2) *gleyic solonetz*—those soils with groundwater influence in the upper 50 cm, and (3) *mollic solonetz*—the soils with a dark colored surface layer, often high in organic matter. Soils with a lower  $\text{ESP}$  than a solonetz, but higher than 6, are mapped as a “sodic phase” of other soil units.

### 2.2.3 USDA: SOIL SURVEY DIVISION STAFF CLASSIFICATION (1993)

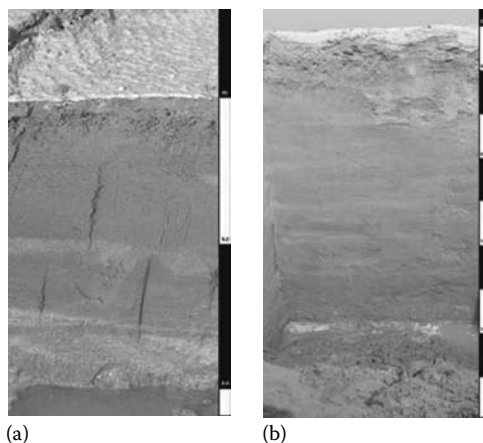
The following classes of salinity are used if the  $\text{EC}$  has not been measured, but salinity is inferred (Soil Survey Division Staff, 1993). These are: class 0 (nonsaline,  $0\text{--}2 \text{ dS m}^{-1}$ ); class 1 (very slightly saline,  $2\text{--}4 \text{ dS m}^{-1}$ ), class 2 (slightly saline,  $4\text{--}8 \text{ dS m}^{-1}$ ), class 3 (moderately saline,  $8\text{--}16 \text{ dS m}^{-1}$ ), and class 4 (strongly saline  $\geq 16 \text{ dS m}^{-1}$ ). The class 0 shows no visible salts on the soil surface and plant growth is not affected by salinity/sodicity. In classes 1 and 2, the plant growth may be uneven or patchy. Salts are generally present in small-sized patches (Plate 2.1a), which do not cover more than 25% area collectively. In class 3, the plant growth on these soils is very patchy and the salts are fairly visible on the soil surface. The area in class 4 lies unused and may support some salt-tolerant plants.

### 2.2.4 USDA-NRCS (KEYS TO SOIL TAXONOMY, 2010) CLASSIFICATION

The Keys to Soil Taxonomy (USDA-NRCS, 2010) system of classification has hierarchies of groups of soils (taxa). In this system, the true salt-affected soils belong to the order “Aridisols” and suborder salids. At the third level of classification, there are great groups named Natrargids, which are argids with a high  $\text{ESP}$  and are equivalent to solonetz on the soil map of the world (FAO-UNESCO, 1974). At this third level (great group) of classification, there are also the aquisalids (Plate 2.2a—salids with water table within 1 m from soil surface) and haplosalids (Plate 2.2b—where water table is below 1 m or even deeper) in the suborder of salids, which are equivalent to solonchaks on the soil map of the world.

### 2.2.5 RUSSIAN SYSTEM OF SALINITY CLASSIFICATION

In the Russian classification, the solonchaks may be “external solonchaks” with the soluble salts throughout the whole soil or internal solonchaks with soluble salts in the subsoil or substratum only. The solonchaks are subdivided according to the composition of salts. The following types have been recognized: nitrate, nitrate-chloride, chloride, chloride-sulfate, sulfate-chloride, sulfate-soda, soda, and borate solonchaks. The external solonchaks are of different types (flooded, puffed, sabkha),



**PLATE 2.2** Soil salinity classes (USDA-NRCS): (a) aquisalids—water table at 40 cm and (b) haplosalids—water table at 140 cm.

sometimes the subdivision is made according to the origin of the salt, e.g., closed basin, marine, allochthonous air blown, and anthropic.

## 2.3 SALINITY ASSESSMENT

Accurate measurement is essential to understand soil salinity problem for better management, to improve crop yield and to maintain root zone soil health. If the salinity could be measured, it could be managed. A reliable salinity assessment method is required. The choice of the method, however, depends on purpose, size of the area, depth of soil to be assessed, number and frequency of measurement, accuracy required, and available resources.

There are a number of soil salinity assessment tools, such as salinity monitor maps prepared over a period of time to assess present salinity problem and to predict future salinity risk to the area, salinity indicators on soil surface, vegetative indicators, conventional salinity tests (EC 1:1 or 1:5; ECe) and modern methods (Geophysical—EM38; salinity sensors).

### 2.3.1 REMOTE SENSING AND SOIL SALINITY

Remote sensing acquires information about the Earth's surface without actually being in contact with it. The fundamentals of remote sensing in soil salinity assessment and examples of such studies from the Middle East, Kuwait, Abu Dhabi Emirate, and Australia have been described recently by Shahid et al. (2010). The remote sensing imagery picks surface reflection and provides general salinity information of the area; however, it lacks information about root zone salinity, which requires other conventional (EC meters) and modern methods (EMI and salinity probes) to be used. The combination of salinity maps taken over period of time and digital elevation model (DEM) help predict salinity risk in the area (Furby et al., 1995, 1998).

### 2.3.2 CONVENTIONAL METHODS

Soil salinity measurement is made on georeferenced (using GPS) field sampling, and laboratory analysis of extract from saturated soil paste (Plate 2.3a) by EC meter is accepted as the standard way of soil salinity assessment, expressed as desisiemens per meter ( $\text{dS m}^{-1}$ ) or millisiemens per centimeter ( $\text{mS cm}^{-1}$ ). This is due to the amount of water that a soil holds at saturation, is related to soil texture, surface area, clay content, and cation-exchange capacity. The lower soil:water ratios (1:1, 1:2, 1:5) are also used in many laboratories; however, the results require calibration with ECe to select salt-tolerant crops.



**PLATE 2.3** Salinity assessment methods: (a) saturation extract collection, (b) salinity surveys by EM38, (c) activity meter and probe, (d) placing sensor in the root zone, (e) buried sensor and smart interface and (f) instant viewing of EC on smart datalogger.

### 2.3.3 MODERN METHODS

The salinity assessment and management at farm level help farmers improve crop productivity. The conventional field sampling and laboratory analysis is a tedious, expensive, and time-consuming process. Other quicker and modern methods can be used in the field salinity mapping, such as electromagnetic induction (EMI-EM38) and activity meter with salinity probe. The EM38 (Plate 2.3b) is most commonly used in agricultural surveys and for rapid assessment of the soil's apparent electrical conductivity (ECa) in millisiemens per meter ( $\text{mS m}^{-1}$ ). The EM38 has transmitter and receiving coils. The transmitter coil induces an electrical current into the soil and the receiving coil records the resulting electromagnetic field. The EM38 provides a maximum of 1.5 and 0.75 m depth of exploration in vertical and horizontal dipole modes, respectively. EC mapping is one of the simplest, least expensive salinity measurement tools. Integration of GIS with salinity data results in salinity maps and help farmers interpret yield variations and in understanding subtle salinity differences across agricultural fields, allowing them to develop more precise management zones and, ultimately, potentially higher yields.

Activity meter with salinity probe (Plate 2.3c) is handy equipment and gives instant apparent electrical conductivity (ECa) information in  $\text{mS cm}^{-1}$  and  $\text{g L}^{-1}$ . The German-made PNT3000 COMBI + model is commonly used in agriculture, horticulture, and landscape sites for rapid salinity assessment and monitoring. It provides an extended EC-measuring range from 0 to 20  $\text{mS cm}^{-1}$  and from 20 to 200  $\text{mS cm}^{-1}$ . The unit includes stainless steel measuring electrode 250 mm long for direct soil salinity measurements; EC-plastic probe with platinum-plated ring sensors and high-quality aluminum-carrying case. The operation is convenient and simple; only one button makes the full operation possible. It is essential to validate ECa values with ECe from same fields. In both cases, the ECa must be correlated to ECe for crop salt tolerance.

The most modern salinity logging system (Plate 2.3d through f) is real-time dynamic automated salinity logging system (RTASLS). In this system, ceramic sensors are buried in the root-zone where salinity monitoring is required. Each salinity sensor is fitted with an external smart interface that consists of an integrated microprocessor containing all the required information to

allow autonomous operation of the sensor, including power requirements and logging interval. The smart interface resolution is 16 bit, offering highly precise and accurate recording of the salinity sensor. The smart interface is connected to DataBus, which leads to Smart Datalogger. The Smart Datalogger searches the DataBus and automatically identifies the number of salinity sensors connected and begin logging them at the predetermined intervals. Instantaneous readings from sensors can be viewed on the logger's display directly in the field without the need for a laptop. Data can also be accessed in the field by memory stick or remotely using a mobile phone modem. This data is then available for graphing and interpretation in Excel (Shahid et al., 2009a).

## 2.4 SOIL SALINITY IN IRRIGATED FIELDS AND RELATIVE YIELD PREDICTION

Crops can tolerate salinity up to certain levels without a measurable loss in yield (this is called threshold level). As a general rule, the more the salt tolerant the crop, the higher the threshold level. At salinity levels greater than the threshold, crop yield reduces linearly as salinity increases. Using the salinity values in a salinity/yield model developed by Maas and Hoffman (1977), predictions of expected yield loss can be made. Maas and Hoffman expressed salt tolerance of crops by the following relationship:

$$Y_r = 100 - s(EC_e - t)$$

where

$Y_r$  is the percentage of the yield of crop grown in saline conditions relative to that obtained on nonsaline conditions

$t$  is the threshold salinity level where yield decrease begins

$s$  is the percent yield loss per increase of 1  $EC_e$  ( $dS\ m^{-1}$ ) in excess of  $t$

Salinity mapping at the farm level and Table 2.1 may be used as a guide to predict yield losses.

## 2.5 SALINITY MANAGEMENT AND RECLAMATION

It is essential to keep the plant root zone salinity below crop threshold level to get higher production and to maintain soil health. This requires careful management and reclamation of irrigated agricultural fields. The main objectives of management and reclamation should be to bring more soils under cultivation, to increase the yield per unit area, and to increase the water and fertilizer use efficiency, and to improve livelihood of the farmers. Efficient, effective, and long-term reclamation of saline soils require the lands to be well leveled before leaching is initiated, additional supply of good quality water is required and good subsurface drainage is essential. The physical, hydraulic, chemical, and biological techniques are the methods of soil reclamation.

### 2.5.1 PHYSICAL METHOD

Physical method includes land leveling, salts scraping, deep ploughing and tillage, subsoiling and sanding. In order to remove salts through leaching or flushing, *leveling* (preferable laser leveling) is a prerequisite to allow uniform distribution of water. The objective is to leach the salts or flush from the surface if a near surface restrictive layer is present. The leveling process may compact the soil due to heavy machinery used, subsoiling or chiseling should follow this practice. In certain cases, salt crusts formed at surface can be removed by mechanical means. In small agricultural farms, salt *scraping* is the simplest and most economic way of reclaiming saline soils. Scraping can minimize

**TABLE 2.1**  
**General Threshold (t) and Slope (s) Values to Calculate Crop Yield**  
**as a Function of Soil Salinity for Various Crops**

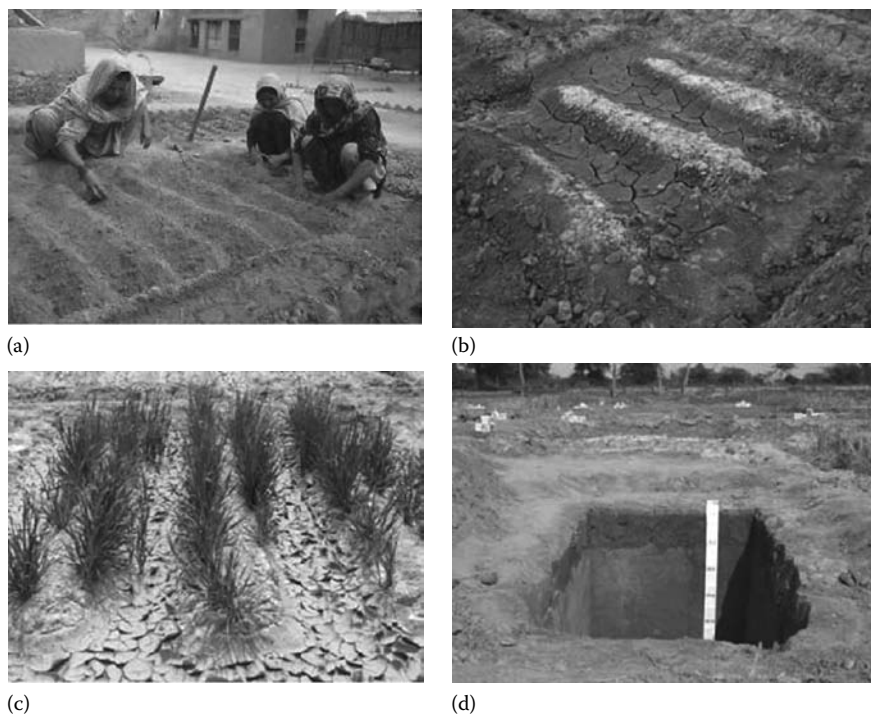
| Crops                                          | Threshold (t) ECe (dS m <sup>-1</sup> ) | Slope (s) % Yield Loss per 1 ECe (dS m <sup>-1</sup> ) above (t) |
|------------------------------------------------|-----------------------------------------|------------------------------------------------------------------|
| Alfalfa ( <i>Medicago sativa</i> )             | 2.0                                     | 7.3                                                              |
| Barley for grain ( <i>Hordeum vulgare</i> )    | 8.0                                     | 5.0                                                              |
| Bean ( <i>Phaseolus vulgaris</i> )             | 1.0                                     | 18.9                                                             |
| Bean, dry edible ( <i>Phaseolud vulgaris</i> ) | 1.0                                     | 19.0                                                             |
| Cabbage ( <i>Brassica oleracea</i> )           | 1.8                                     | 9.7                                                              |
| Carrot ( <i>Daucus carota</i> )                | 1.0                                     | 14.1                                                             |
| Clover ( <i>Trifolium</i> spp.)                | 1.5                                     | 12.0                                                             |
| Corn for grain ( <i>Zea mays</i> )             | 1.7                                     | 12.0                                                             |
| Corn for silage ( <i>Zea mays</i> )            | 1.8                                     | 7.4                                                              |
| Cucumber ( <i>Cucumis sativus</i> )            | 2.5                                     | 13.0                                                             |
| Date ( <i>Phoenix dactylifera</i> )            | 4.0                                     | 3.6                                                              |
| Lettuce ( <i>Latuca sativa</i> )               | 1.3                                     | 13.0                                                             |
| Onion ( <i>Allium cepa</i> )                   | 1.2                                     | 16.1                                                             |
| Pepper ( <i>Capsicum annum</i> )               | 1.5                                     | 14.1                                                             |
| Potato ( <i>Salanum tuberosum</i> )            | 1.7                                     | 12.0                                                             |
| Radish ( <i>Raphanus sativus</i> )             | 1.2                                     | 13.0                                                             |
| Sorghum for grain ( <i>Sorghum bicolor</i> )   | 6.8                                     | 16.0                                                             |
| Soybean ( <i>Glycine max</i> )                 | 5.0                                     | 20.0                                                             |
| Spinach ( <i>Spinacia oleracea</i> )           | 2.0                                     | 7.6                                                              |
| Sugar beet ( <i>Beta vulgaris</i> )            | 7.0                                     | 5.9                                                              |
| Tomato ( <i>Lycopersicum esculentum</i> )      | 2.5                                     | 9.9                                                              |
| Wheat for grain ( <i>Triticum aestivum</i> )   | 6.0                                     | 7.1                                                              |

Source: Hoffman, G.J., Water quality criteria for irrigation. Biological System Engineering University of Nebraska, Institute of Agricultural and Natural Resources. Publication No. EC 97-782, 2001.

Notes: s, % yield loss per 1 ECe (dS m<sup>-1</sup>) increase above t (ECe) value; t, salinity threshold ECe (dS m<sup>-1</sup>), where yield is optimum.

the salts temporarily; however, they can reappear with a continuous feed of ground water to the surface. Low salinity in the rootzone can be achieved through *tillage* practices by manipulating the soil surface condition, i.e., bed shape and irrigation management (Plate 2.4a). It is very well recognized that salts tend to accumulate on the ridges top away from the wet ridge shoulder (Plate 2.4b) when furrow irrigation is adopted. Placing the seeds on off-center slope of the single row will put the seed (Plate 2.4a) in minimum salinity and optimum moisture condition. Under high salinity, the alternate row should be left unirrigated; this will ensure maximum accumulation of salts in the unirrigated area and leave the irrigated furrows free of salts and fit for planting seeds (Plate 2.4b and c). *Subsoiling* is particularly important for disrupting the dense layers (Plate 2.4d) at depth to enhance permeability (Shahid et al., 2009b). In the absence of subsoiling, flushing should be preferred over leaching; the latter compounds the salinity problem in the root zone due to the dense layer. Subsoiling is important while reclaiming sodic soils after the addition of a suitable amendment such as gypsum and watering the field.

If the soil surface to be reclaimed is very heavy textured, mixing of sand, “sanding,” to the surface can change the texture permanently, and the soils become more permeable and easy to reclaim. This practice also provides a favorable environment for plant growth compared to the original soil without sanding.



**PLATE 2.4** Seed bed, salinity development, and plant growth: (a) Seed placement on furrow shoulder, (b) salt accumulation on ridge tops, (c) barley plants growing on ridge shoulder, and (d) dense layer need subsoiling.

### 2.5.2 HYDROLOGICAL METHOD

Hydrological method is concerned with water use and drainage. In irrigated agriculture, the objective is to free the root zone from salts through leaching to lower depths and the subsequent drainage and surface flushing of dissolved salts. The rootzone salinity may increase if the net downward movement of salts is less than the salt input from irrigation, and salt water flux to surface. Therefore, salt balance must be kept under control, and this is a function of irrigation water salinity and to the success of drainage system.

Traditionally, saline soils have been reclaimed by flooding or by ponding water. In general, the depth of soil leached is roughly equal to the depth of water infiltrated during leaching. In order to leach the salts, the leaching requirement (LR) is very important. The LR is the calculated fraction (depth) or quantity of water that must pass through the rootzone to maintain the EC of the drainage water at or below some specified level. The recent trend is to minimize this LR in order to prevent raising the groundwater and minimize the load to drainage system (Mashali, 1995). Methods of LR calculation and to predict the losses in yield due to salinity are described by Rhoades (1992).

Timely leaching is important to assure root zone salinity is not exceeded above crop salinity tolerance limit for extended periods of time or critical stage of plant growth. In normal conditions, leaching can be accomplished at each irrigation; however, in soils with low infiltration rate and for crops sensitive to excess moisture in the rootzone, leaching at each irrigation may not be appropriate. Leaching can be done when soil moisture is low and water table is deep; it should precede the critical growing stage; at low evapotranspiration demand; at night, during high humidity, in cooler weather and; at the end of cropping season, as appropriate to area.

The drainage lowers water table, provides adequate leaching, minimizes upward water flux, and thus controls salinity buildup. Provided the subsurface is permeable and relief is adequate, natural



drainage may work; however, experience shows that such ideal conditions do not prevail in saline areas, and therefore, a drainage system is always required. Based on site condition, nature of the problem, and available resources, a suitable drainage system (surface or subsurface) can be selected. Surface drainage allows runoff excess water before entering to soil and subsurface drainage is used to control water table at safer depth, consisting of open ditches or tile drains or perforated plastic pipes, mole drainage, and vertical drainage (pumping water) when the deep horizons have an adequate hydraulic conductivity.

2.5.2.1 Modern Irrigation Methods

In arid and semiarid zones, the major constraints are limited quantities of good quality water and to increase its efficiency; and exploitation of unsuitable brackish/saline water for irrigation. Therefore, a suitable irrigation method is to be selected without invoking soil salinity hazards. Each irrigation system develops salinity at a specific soil zone that is to be carefully monitored.

*Surface* irrigation includes flood, basin, border, and furrow methods. At the end of each irrigation cycle, soil dries out concentrating the salts, which adversely affects the crop yield. Frequent irrigation may lower the salinity, but increase the wastage of water; the alternatives to improve the efficiency of water are the drip, subsurface, or sprinkler irrigation. This shift from conventional surface irrigation to modern irrigation is costly and requires assurance on better crop adaptability.

A good *sprinkler* irrigation must meet the requirements of the crop for water (ET). It often allows efficient and economic use of water and reduces deep percolation losses. If water application through sprinkler is in close agreement with crop needs (ET and leaching), drainage and high water table problem can be greatly reduced, which in turn should improve salinity control. The use of high salinity water may lead to leaf burn and, therefore, the quality of water must match with the leaf burn tolerance of plants. Under sprinkler irrigation, the net salinity built up is at subsurface.

The *drip* (trickle) irrigation supplies the required quantity of water to the crop almost on a daily basis. The poor quality water used in drip irrigation may yield better due to continuous high moisture contents and daily replenishment of water loss by ET. Drip has priority over sprinkler as the latter may cause leaf burn, defoliation of sensitive species, which is not the case with drip irrigation. Maximum salt accumulation is outside the edges of the area wetted by emitters (Plate 2.5).

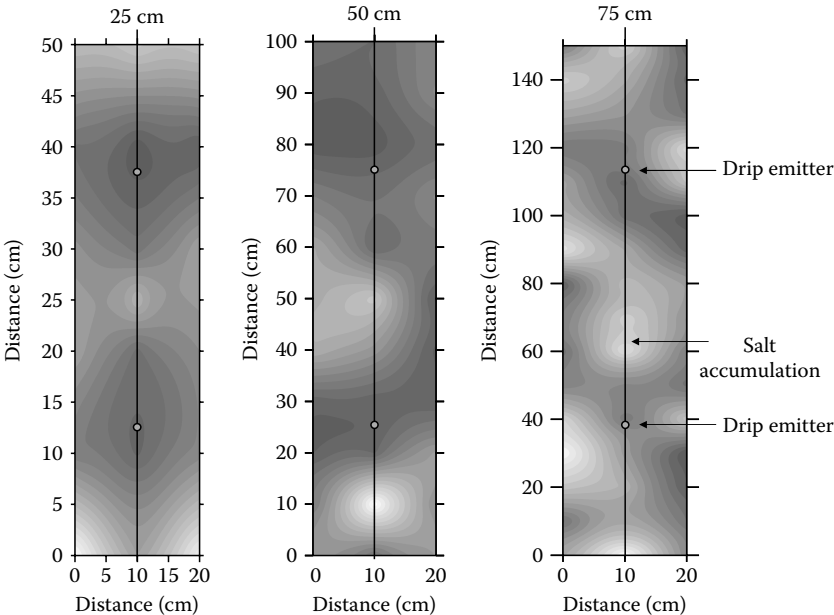


PLATE 2.5 Drip irrigation and salt accumulation (salinity map).

The daily irrigation continuously moves the moisture down to keep the salts under control. Plants may get shock due to high salinity when rainfall, as the rain water pushes the salts from edges to central rootzone; it is advisable to not shut the drip irrigation during rainfall to push the salts continuously toward the edges of the area wetted by emitters.

*Subsurface drip irrigation* (SDI) compared with other irrigation systems SDI reduce water losses to evaporation, deep percolation, and completely eliminate surface runoff (Phene, 1990), increase crop marketable yield and quality (Ayers et al., 1999), and can result in high nutrient use efficiency (Thompson et al., 2002). Saline irrigation water can be used with SDI, while maintaining yields and improving water use efficiency compared to surface irrigation (Tingwu et al., 2003; Cahn and Ajwa, 2005) because SDI can result in suitable root-zone salinity. The limitation of SDI is that salts continuously build up at surface through capillary action above the buried drip lines during growing season (Oron et al., 1999) and therefore the concept of LR does not work under SDI; however, salts above buried drip (surface salinity) can be managed by supplementing with sprinkler irrigation (Thompson, 2010). This approach may be costly, but a compromise.

### 2.5.3 CHEMICAL METHOD

Chemical methods are used to reclaim sodic soils. To have successful crops on sodic soils, ESP of the soil must be below threshold ( $<15$ ). The main aim is to increase the concentration of calcium in the soil. The long-term objective is to replace ES with calcium, and use organic matter to bind the soil and improve its structure. Gypsum ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) is commonly used amendment (to supply Ca) to rectify sodicity problem in irrigated fields. Calcium causes particles to form clusters (floculates), forming a very clear puddle of water. Gypsum addition changes the soil chemistry in two ways: (1) by increasing salt solution clay do not swell and disperse. This is a short-term effect, which occurs as the gypsum dissolves; (2) the calcium from gypsum replace the ES attached to the clays (exchange complex). The process changes a sodic clay to a calcium clay, making it less prone to swelling and clay dispersion. The displaced sodium cations are leached below the plant root zone. Mined gypsum (10 mesh) is commonly used.

The addition of HCl and  $\text{H}_2\text{SO}_4$  is only recommended where soils are sodic and calcareous. The acid induces Ca from  $\text{CaCO}_3$  and Ca released work in similar manner as from gypsum. Acids are highly corrosive and dangerous to handle; special equipments are available to apply acid in the field and usually it is applied with irrigation water.

Addition of elemental sulfur may also be useful in reclaiming sodic soils, with the condition that it is properly oxidized. The sulfur (S) may be oxidized through biological oxidation by *Thiobacillus thiooxidans*, in sodic soils it is very slow process. The final product is  $\text{H}_2\text{SO}_4$ .

### 2.5.4 BIOLOGICAL METHOD

#### 2.5.4.1 Serial Biological Concentration of Salts

The concept of serial biological concentration of salts (SBCS) (Heuperman, 1995) may provide an alternative salt management option, especially where groundwater pumping and safe disposal is an issue. The objective of SBCS is therefore to lower water table and reduce water volume. The SBCS system involves reuse of drainage water on progressively increasing salt-tolerant crops (salt-sensitive crops, salt-tolerant crops, halophytes, trees, etc.). In SBCS cropping system, each crop is underlain by tile drain for the collection of water to be used to irrigate the next stage. Along the crop sequence, the volume of drainage water collected is reduced due to plant water use, and the salinity of the drainage water increases since there is little or no salt uptake by plants. The highly saline water can be either transferred to treatment plants where through reverse osmosis or other techniques, it can be treated and salts can be removed, or the final effluent is contained in relatively small evaporation ponds making it feasible to consider the use of floor lining to eliminate leakage. These ponds

could be used for fish farming. The highly saline water may be collected in a series of ponds where through evaporation salts may be harvested, and may have commercial value such NaCl (halite) for caustic soda factories and other uses.

#### 2.5.4.2 Biosaline Agriculture (Practicing Salt-Tolerant Crops)

Biosaline agriculture means economic utilization of salt-affected soils, saline/brackish waters for agricultural purposes. It involves cultivation of salt-tolerant species of agricultural significance and adoption of special agronomic practices to improve their productivity under such conditions. Some scientists use saline agriculture concept, but “biosaline” is much broader in scope and includes manipulation of desert and sea resources for food and fuel (energy) production. Tables for salt tolerance of different crops are reported elsewhere (Maas, 1990).

For practical biosaline agriculture, the very first step is to identify the nature of the problem and then to visualize appropriate measures for maximum economic returns under the specific situation. Biosaline agriculture has many dimensions such as selection/breeding of salt-tolerant genotypes and plant species; domestication of salt-tolerant wild plant species for economic exploitation of salt-affected lands; introduction into the cultivated crops/other plant species genes of salt tolerance from their wild relatives through genetic engineering; agronomic practices including methods of land preparation, planting, irrigation and fertilizers application; and physiological studies with the following objectives. Other aspects in biosaline agriculture are; (1) to determine critical plant factors controlling yield under saline conditions, (2) to study physiological differences between salt-tolerant and salt-sensitive genotypes with a view to develop selection criteria for salt tolerance, (3) to improve yield through special treatments at critical stages during plant growth, and (4) the use of salty water for agricultural purposes. The Dubai based International Center for Biosaline Agriculture is specialized in such studies and have developed various production systems and introduced in many countries (Pakistan, UAE, Oman, Tunisia, Syria, Jordan, Palestine, Uzbekistan, etc.) for saline soils.

#### 2.5.5 ALTERNATIVES FOR USING MARGINAL SALINE LANDS

There are persuasive reasons to extend the range of agricultural production into more saline environment. Some of these areas are uneconomical to reclaim by conventional soil reclamation procedures, e.g., leaching of salts and their drainage and use of chemical amendments. On such areas, the management and improvement of wild stands of *Atriplex* (salt-bush) for grazing have been pursued for many years in Australia. Now, there is increasing interest in cultivating, breeding, and managing selected *Atriplex* species for intensive forage production with highly saline water (Plate 2.6a).

Halophytes are one choice for such soils; they can grow under very saline conditions, for example, *Juncus vigidus* and *J. acutus* can grow in saline marshes or under irrigation with brackish water



(a)

(b)

**PLATE 2.6** Alternative use of saline lands: (a) *Atriplex* growing in inland salt flat (sabkha) and (b) coastal mangroves in Abu Dhabi Emirate.

or even seawater. The culms provide fibers for high-quality paper production and the seeds have the medicinal value. The *Leptochloa fusca* (kallargrass) as forage, green manuring, compost, crop, pulp for paper production is found to be salt-tolerant in almost any salt-affected soils in most areas even irrigated with highly sodic water. Mangroves grown in coastal areas (Plate 2.6b) are other options; they provide fuel and fodder and deserve more attention for planting along shorelines. The sites may also be used for recreation purposes.

## 2.6 ECONOMIC, ENVIRONMENTAL, AND SOCIAL LOSSES DUE TO SOIL SALINITY

Economic losses are faced through loss of agricultural production and costs are incurred for land rehabilitation for public utilities.

Environmental losses are introduced through land degradation by physical, chemical, and biological changes in the soils and waterways, loss of vegetation and change to the landscape.

Social losses include an increased production costs on farms and reduced value of land as a result of soil salinization. The person from the salinized area move away and the social set up is disturbed.

## 2.7 RESEARCH–EXTENSION–FARMER LINK

There should be strong link of research–extension–farmers to benefit the end chain member (farmers, the stakeholders). Therefore, education to farming community is vital in increasing awareness and understanding of salinity. Advisory program should be developed to help farmer's plan and use salinity control practices on their farms. Salinity mapping on whole farm scale is the best practice for crop selection. Farmers should realize the significance of salinity mapping for the sustainable use of their farms. Salinity exhibitions for community education should be arranged in government institutes; demonstration days at the farmer's field are also useful. Preparation of introductory brochures for salinity control and management at the farm level and their distribution to the farming community can enhance their understanding to tackle salinity in a sustainable way. The awareness of the problem to teachers gives students hands-on experience and helps them discuss options with their students. After all, today's students will be tomorrow's soil resource users and managers.

## 2.8 SUMMARY

Salt-affected soils are a major global issue owing to their adverse impact on agricultural productivity and sustainability. Soil salinity/sodicity undermines the resource base by decreasing soil quality. This could be natural or a symptom of misuse and mismanagement that jeopardizes the integrity of soil's self-regulatory capacity. Recent estimates show that salt-affected soils occur throughout the world. The countries affected by salinization are predominantly located in arid and semiarid regions where continued irrigation with low-quality groundwater has contributed the expansion of salt-affected soils. In order to exploit these soils to their full potential, there is a need for a sound basis to optimize their use, determine their potential, productivity, and suitability for growing different crops, characterize these soils, and identify appropriate integrated reclamation and management practices. Management and reclamation can only be achieved if causes rather than the symptoms are identified and controlled. In this chapter, technologies for the characterization, reclamation, and management of salt-affected soils are described. In addition, different classification systems of salt-affected soils adopted in the world as well as soil reclamation technologies including hydraulic, physical, chemical, and biological approaches are described. Moreover, some possible alternatives to utilize the salt-affected soils are also discussed. Finally, link between research–extension–farmers has been proposed. The chapter demonstrates an easy format and language that is designed to be user-friendly.

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# 3 Soil Salinization and Management Options for Sustainable Crop Production

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## 3.1 INTRODUCTION

### 3.1.1 FOOD PRODUCTION AND IRRIGATED LAND

The dramatic increase in total global food production over the last 50 years has avoided the predictions of large-scale food famine. In addition to the increase in cultivated land, there has also been an increase in crop production on a per-acre basis. This increase is generally attributed to the development of improved crop varieties and management practices (green revolution); however, an important part of this increase is related to an increase in the amount of irrigated acreage. Irrigated lands have much higher productivity and economic return per acre as compared to nonirrigated lands. For example, it is estimated that globally, irrigated lands represent 15% of the cultivated land, yet they produce over 30%–40% of the world's food (Ghassemi et al., 1995; Postel, 1999). In arid regions, the impact of irrigation is much greater than the rest of the world in general. Arid regions have low production in the absence of irrigation due to insufficient water to meet crop needs. Also, most arid lands are located in high-temperature environments, and, therefore, with adequate water they can be almost continually cropped, with multiple harvests. The 35% increase in irrigated land from 1970 to

the late 1980s thus provided a significant part of the increase in world food production and was a major factor in avoiding large-scale famine.

Since the 1980s, there has been a decline in the rate of growth in the world's irrigated land. By the start of the twenty-first century, total irrigated acreage reached a constant value. The stabilization in total irrigated acreage is due primarily not to lack of additional suitable arable land or financial resources, but rather to the lack of new developable water supplies in most of the arid and semiarid regions that can most benefit from irrigation.

The current levels of irrigation in arid regions are clearly not sustainable with existing water supplies. Supplemental irrigation in more humid regions has increased, masking the actual decline in irrigated acreage in arid regions. Globally, irrigated agriculture uses approximately 65% of the total fresh water, with the industrial and municipal sector making up the balance. California in the United States has experienced significant declines in irrigated acreage. For example, during the 2009 irrigation season, over 180,000 ha were taken out of irrigation in the Central Valley, with the likelihood that there will not be sufficient water in the future to bring this land back into production. "Land banking" is occurring in other irrigation districts, such as Palo Verde and Imperial, where long-term contracts have been signed in transferring former irrigation water to municipal water entities. Additional declines in irrigated acreage are occurring due to the partial restoration of natural water flows for environmental considerations. Future declines are anticipated due to declining ground water supplies as well as increasing urban and environmental water demands. Currently, the percentage of fresh water used by agriculture in California has declined from 75%, as recently as 20 years ago, to below 50%, with a corresponding increase by percentage used by the municipal sector and a decrease in overall use.

Unfortunately, most arid regions do not have new developable surface waters, and the current large fresh water extractions of ground water required for irrigated agriculture cannot be sustained let alone increased. This overutilization of fresh water, extracting what is often called fossil water, is particularly severe in drier regions of the world, where population density, poverty, and food demands are greatest. Overdrafting of ground water has resulted in declining water tables, loss of shallow fresh water for municipal use, and sea water intrusion in coastal regions. In the early 1990s, approximately one-fifth of the irrigated lands in the United States were extracting groundwater in excess of their natural recharging capacity (Postel, 1997). The data are not completely known, but the situation appears more severe in many less developed nations in arid and semiarid regions.

An increasing population results in increasing total demand for fresh water for municipal and industrial use as well as for increased food production. Increased fresh water needs are also related to increased per capita water usage associated with improved economic conditions in a region. Increases in living standards are not only related to increased domestic per capita water consumption, but they also result in increased water consumption related to food production on a per capita basis. This increased demand with improved living standards is related to the increased water requirement for meat production versus grain production (expressed as gallons or liters of water per kcal). It will be a major challenge just to maintain the existing level of irrigation and associated food production in the arid and semiarid regions of the world. An increase in living standards will require yet more water.

## 3.2 SALINITY

### 3.2.1 EXTENT OF SALINITY PROBLEM

It is estimated that there are 76 million hectares (Mha) of human-induced salt affected land, representing 5% of the world's cultivated land (Ghassemi et al., 1995). Salt affected lands are those where crop yields are reduced or where less desirable crops must be grown because of the salinity. This human induced salinization is termed secondary salinization, in contrast to regions that were saline



in their native condition. This value underestimated the extent of salinity because it does not include large areas of land that could be potentially cultivated, if not for the native salinity.

Salinity problems are most prevalent in irrigated lands relative to the total cultivated acreages. This is not surprising as irrigated lands are concentrated in more arid regions, where salinity is more likely to be a problem. Also, irrigation results in the application of additional water to the landscape, thus imposing additional drainage needs to the natural hydrologic system. Of the world's 227 Mha of irrigated lands, it is estimated that 45.4 Mha, or 20%, are adversely impacted by secondary salinization (Ghassemi et al., 1995).

Salinity is a major threat to current irrigation projects and to the remaining near-surface fresh water supplies in arid regions. The extent of the salinity problem has not stabilized; instead, it is estimated that as much as 2 Mha of irrigated land, representing approximately 1% of the total, is lost from production due to salinity each year (Umali, 1993, in Postel 1997). Most of the world's salt affected, cultivated lands are in Asia and Africa, where population densities and economic conditions make the problem proportionately more severe. For example, it is estimated that Egypt, Iran, and Pakistan had 33%, 30%, and 26%, respectively, of their irrigated land impacted by secondary salinization (Ghassemi et al., 1995). Developed countries are not immune to these salinity problems. For example, it is also estimated that over 20% of irrigated land in the United States is salt affected (Postel, 1999), a value comparable to the global average.

### 3.2.2 MANAGEMENT IMPACTS

In contrast to salinization of water supplies, soil salinization is generally more readily controlled. Most soil salinization has historically occurred as a result of over-irrigation. For earlier civilizations, this can be partially attributed to lack of knowledge concerning water use or requirements relative to quantities of water applied.

In the past two centuries, over-irrigation and salinization can mostly be attributed to two factors: the design and operation of irrigation projects, and overemphasis on the need for leaching. Irrigation projects have been designed without sufficient coordination between plant scientists, irrigation scientists, and civil and hydraulic engineers. Irrigation specialists, focused on the development of new irrigation projects, have emphasized the need to leach salts out of the root zone to enable maximum yields. The concept was that salts had to be "pushed" down into the profile to avoid surface salinization and crop failure; the more leaching the better.

With initially abundant water, older irrigation systems were typically developed with earthen canals and laterals, and irrigation was by furrow or wild flooding, resulting in nonuniform water application. The limitations of these irrigation systems, combined with the overemphasis on leaching, have resulted in low irrigation efficiency. In many, if not most, instances the large delivery system water losses and excessive water applications result in large drainage volumes to the subsurface, in excess of natural drainage capabilities. Excessive drainage volumes, in turn, usually results in subsequent water logging, evaporation of water from the surface, and deposition of salts at or near the soil surface in low lying parts of the irrigation district. Expensive drainage systems are subsequently often constructed, controlling the root-zone salinity but discharging large volumes of saline water to the drainage system, typically causing adverse salt impacts to downstream users.

Increased salinization in arid and semiarid regions is also often caused by leaching of existing salts from the soil during irrigation in regions containing strata with high salt, as well as by application of waters of low quality without proper management. In the instance of soils high in native salts, regional salinization of ground and surface waters is aggravated by excessive water applications. The impacts of leaching salts present before irrigation may be observed for over a period of 120 years after initiation of the irrigation project (Grand Valley, Colorado), depending on the hydrology of the system, type of salts present, and depth and design of the drainage system, if present.

### 3.2.3 SALINIZATION OF WATER RESOURCES

In addition to the unsustainable extraction of fresh water, there is a related decline in water quality of existing supplies; thus, these factors are not unrelated. There are two general factors contributing to the decline in water quality. Extraction of fresh water from a system reduces the extent of dilution of other natural or man-induced salt loads. Second, as irrigation brings more salts into a valley, it adds a new source of drainage of additional saline water into the receiving body of water. These concentrated drainage waters contain both the initial salts present in the irrigation water as well as salts already in the soil that are displaced by the water leaving the root zone. Thus, in arid regions, irrigation or even changes in cropping patterns that impact recharge often mobilizes salts that have accumulated over geologic time either in the unsaturated zone, salinizing groundwater (Australia), or displacing saline groundwater into rivers (Grand Valley, Colorado and the Colorado River). Again, due to the long flow paths, this additional salt load can continue for an additional 150 year, consistent with hydrologic model predictions.

Salinity increases in the drainage water relative to irrigation water are inevitable. Plants extract water, preferentially, thus concentrating these salts in the remaining soil water. Typically, plants contain only 5%–10% of the salt associated with the volume of water that they extract. Hence, more efficient irrigation (generally resulting in less water applied more uniformly), while desirable, results in smaller volumes of drainage water, but of greater salinity. The salinity increase is approximately inversely proportional to the change in volume (inverse to volume of irrigation water/volume of drainage water). Salinization of water resources, especially ground water, is a slower process than soil salinization, but is much more difficult to remediate.

## 3.3 WATER QUALITY CONSIDERATIONS

### 3.3.1 MEASURING AND REPORTING SOIL SALINITY

It is logical that salinity be reported in terms of concentrations of salts, either as total dissolved solids (TDS) in units of mg/L or g/L or as molar quantities, mol/L. From the perspective of plant response, it is generally considered that the adverse effect is related to the osmotic pressure (OP) of the soil solution. However, it is most convenient to measure the specific electrical conductance (EC), typically reported in dS/m at 25°C. As can be expected, the EC is highly correlated with the concentration of total salts, expressed in mol/L or mmol/L of charge. The widely used approximation

$$\text{TSS (mmol}_c\text{/L)} = 10 \text{ EC (dS/m)} \quad (3.1)$$

where TSS, the total soluble salts (U.S. Salinity Laboratory Staff, 1954), is useful for low to moderate salinity (<5–7 dS/m) as the relationship depends on concentration. Alternatively, the relationship

$$\log \text{ TSS} = 0.990 + 1.055 \log \text{ EC} \quad (3.2)$$

(Marion and Babcock, 1976) can be used, but it is still only an approximate, as the relationship between EC and salt concentration also depends on the ion composition of the salts. A more detailed and accurate estimation than provided by these equations, such as that developed by McNeal et al. (1970), requires knowledge of the solution ion composition for prediction of EC.

As the salinity of water increases upon concentration by the processes of evaporation and transpiration, the relative proportions of sodium and chloride increase due to solubility considerations. The most important minerals affecting solution composition are calcite and gypsum, controlling calcium, bicarbonate, and sulfate ions concentrations. Additionally, various salts, including magnesium silicates (sepiolite) and sodium sulfates, may precipitate at very elevated salinity, depending on the composition of the initial solution.

Calculation of mineral solubility requires calculation of ion activity coefficients, ion pairs, and complexes in order to obtain ion activities. The calculation methods are not presented here, as accurate calculation requires a computer model, of which many are readily available. The major ions in saline waters in general order of occurrence are  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{NO}_3^-$ , and  $\text{K}^+$ .

Often soil extracts are prepared to measure soil salinity and solution ion composition. Addition of water inevitably changes the solution composition by mineral dissolution and cation exchange. Thus, the desired extract from the chemical consideration is the one with the lowest addition of water that can result in water extraction. The saturation extract (U.S. Salinity Laboratory Staff, 1954) is generally used as it results in relatively low addition of water and allows for estimation of soil solution EC at field capacity, since there is an approximate ratio of soil solution at field capacity to extraction paste water content. Other extractions in common use include the 1:1 and 1:2 soil: water ratio extracts. Although these are much easier to prepare, they result in greater dilution of the soil solution. In addition, these extracts have a more variable ratio of field capacity water content/extract water content, as compared to the ratios for saturation extracts.

Prediction of the EC, OP, and solution composition with changing water content upon dilution can be made with the Extract Chem model (Suarez and Taber, 2007), which considers mineral equilibrium, cation exchange, and B desorption. The model can be utilized to convert/extract data from one water content to another (such as converting 1:1 extract concentration data into saturation extract concentration data). This conversion is important in the interpretation of salt-tolerance data, as different reference water contents are utilized in these studies.

### 3.3.2 SODIUM AND pH EFFECTS ON SOIL PHYSICAL PROPERTIES

Waters of increased salinity inevitably contain greater proportions of Na and to a lesser extent Mg relative to Ca, due to solubility considerations. The adverse effects of sodium on soil saturated hydraulic conductivity, (McNeal and Coleman, 1966; Shainberg et al., 1981; Suarez et al., 1984), clay swelling, (McNeal et al., 1966), and clay dispersion and flocculation (Frenkel et al., 1978; Goldberg et al., 1988; Suarez et al., 1984) are well documented. The adverse effect of sodium on soil physical properties is a major concern when using waters of lower quality for irrigation.

The SAR (sodium adsorption ratio) is defined as

$$\text{SAR} = \frac{\text{Na}^+}{(\text{Ca}^{2+} + \text{Mg}^{2+})^{0.5}} \quad (3.3)$$

where concentrations are in mmol/L. Inspection of Equation 3.3 indicates that SAR increases with increasing salinity due to the square root term for divalent ions in the expression, as well as the earlier mentioned increase in the proportion of  $\text{Na}^+$  ions with increasing salinity. The SAR is related to the exchangeable sodium percentage (ESP) in the soil by the following expression:

$$\frac{[\text{ESP}]}{[100 + \text{ESP}]} = k'_g \text{ SAR} = \text{ESR} \quad (3.4)$$

(U.S. Salinity Laboratory Staff, 1954), where  $k'_g$  is a cation selectivity coefficient, typically assigned a value of  $0.015 \text{ (mmol/L)}^{-0.5}$ , thus irrigation with more saline waters almost always results in increased exchangeable sodium.

In addition to direct determination of the SAR of irrigation water, it is important to consider the resultant SAR in the soil solution. The SAR depends on the calcium concentration in solution, which in turn depends on the solubility of calcium carbonate. Ground waters are often equilibrated

at carbon dioxide levels 10–50 times greater than atmospheric. Upon degassing of previously calcite saturated water, calcite precipitates and the calcium concentration in solution decreases. The adjusted SAR concept was developed to consider the change in calcium concentration upon equilibration of irrigation water to earth surface conditions.

The simplest and relatively accurate way to calculate an adjusted SAR is to utilize the following equation (Suarez, 1981):

$$\text{SAR}_{\text{adj}} = \frac{\text{Na}_{\text{iw}}}{\sqrt{(\text{Mg}_{\text{iw}} + \text{Ca}_{\text{eq}})}} \quad (3.5)$$

where  $\text{Ca}_{\text{eq}}$  is the Ca concentration (in mmol/L) in equilibrium with calcium carbonate in the soil. For predictive purposes, we utilize a value that is threefold greater than calcite solubility, since this value is the mean value determined for waters below the root zone (Suarez, 1977). The values of adjusted SAR can be obtained directly from computer models such as Extract Chem. (Suarez and Taber, 2007) or  $\text{Ca}_{\text{eq}}$  can be obtained from Tables in Suarez (1981) or by the following equation (Lesch and Suarez, 2009):

$$\text{SAR}_{\text{adj}} = \frac{\text{Na}_{\text{iw}}}{\sqrt{\text{Mg}_{\text{iw}} + 0.215X(P_{\text{CO}_2})^{1/3}}} \quad (3.6)$$

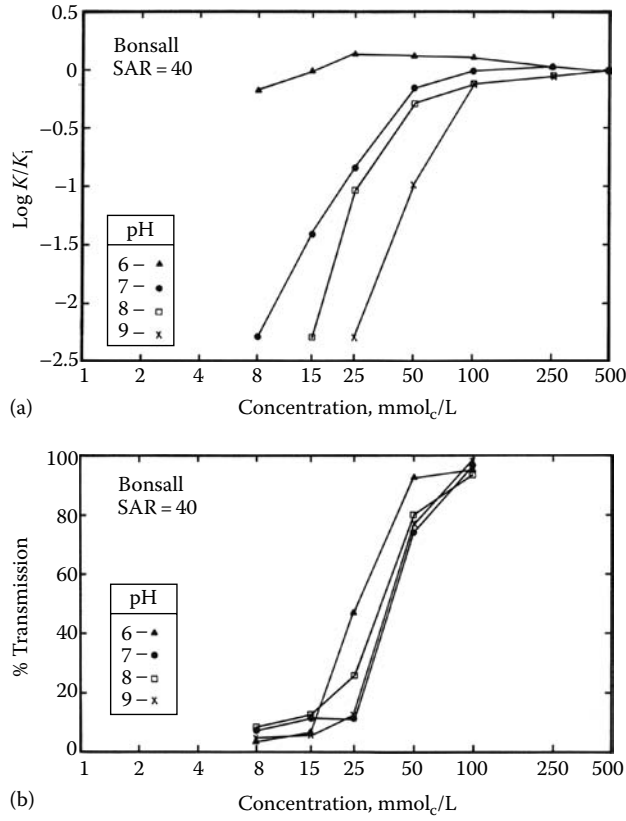
where  $X(P_{\text{CO}_2})^{1/3} = \text{Ca}_{\text{eq}}$ . The  $X$  value considers the equilibrium constants and  $\text{Ca}/\text{HCO}_3$  ratio. The  $P_{\text{CO}_2}$  of surface waters can be represented by the value of  $10^{-3.14}$ , or twice atmospheric.

It is generally considered that the elevated SAR associated with saline waters is not of concern, since the infiltration of these waters is not adversely impacted according to guidelines for sodium hazard (Ayers and Westcot, 1985). However, their analysis does not consider the impact of rain, which results in a rapid decrease in soil salinity at the surface, with a much slower reduction in the exchangeable Na. Computer simulations of changes in exchangeable sodium upon rain on a sodic soil (Suarez et al., 2006) confirm the observed decrease in infiltration that is observed in studies with cyclic rain and irrigation events over an irrigation season (Suarez et al., 2006, 2008). Thus, even in regions where rainfall is an insignificant contribution to the water budget, the dispersive effect of sodium is a significant concern and generally indicates the need to apply a surface soil amendment (such as gypsum) when using waters with  $\text{SAR} > 3$  for irrigation.

The pH of the irrigation water is an important factor related to soil physical properties and infiltration, independent of SAR. Almost all soil hydraulic conductivity studies examining water composition effects utilized chloride as the anion, thus  $\text{pH} < 7$ . Increasing pH also enhances clay dispersion and reduces saturated hydraulic conductivity (Suarez et al., 1984). As shown in Figure 3.1a (Suarez et al., 1984), the relative hydraulic conductivity of an arid land soil at constant  $\text{SAR} = 40$  decreased with decreasing solution concentration. At pH 6, the hydraulic conductivity decreased slightly, while with a further increase in pH the hydraulic conductivity progressively decreased. Related results were obtained from measurements of optical transmission of solutions of soil clay and water that were measured after some fixed times. These measurements represent the process of clay flocculation, as shown in Figure 3.1b (Suarez et al., 1984). Upon comparison of Figure 3.1a with Figure 3.1b, we conclude that although the flocculation tests are simple and quick to perform and provide a useful indication of potential soil dispersion, they do not duplicate the response of the more important hydraulic properties of the soil.

### 3.3.3 WATER QUALITY CRITERIA RELATED TO SOIL PHYSICAL PROPERTIES

There are many factors that relate to soil stability. From the point of view of irrigation of arid land soils, the focus has been on the electrical conductivity (or salt concentration) and the SAR of the irrigation water. The EC-SAR guidelines, presented in a FAO publication by Ayers and

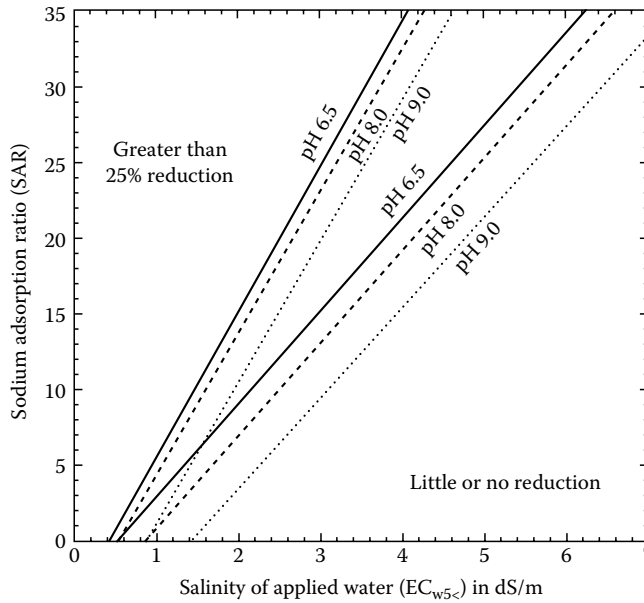


**FIGURE 3.1** (a) Relative hydraulic conductivity and (b) optical transmission of Bonsall soil as related to electrolyte concentration and pH. The saturated hydraulic conductivity was recorded from step-wise reductions in solution concentration at constant SAR. (After Suarez, D.L. et al., *Soil Sci. Soc. Am. J.*, 48, 50, 1984.)

Westcot (1985) have been widely used, generally without consideration of the uncertainty associated with the guideline. The representation presents two stability lines, one for severe effects and the other for slight to moderate reduction in infiltration. Waters with SAR less than the point on the line but at the same concentration or with EC greater than the point on the line, but with the same SAR, were deemed safe to use. However, there is considerable uncertainty as the water hazard depends on many other factors, including pH, organic matter content, Fe and Al oxide content, clay mineralogy, tillage, irrigation system (sprinkle, flood, drip) previous tillage, initial infiltration rate, etc. As shown by Pratt and Suarez (1990), there is a large variability associated with the represented stability line, although there is a good relationship between stability and SAR-EC for any specific experiment.

One of the important factors is the interaction of rain with the irrigation water. It is not sufficient to average the water compositions, as the input of rain causes a rapid decrease in the surface soil EC and a correspondingly smaller decrease in the SAR, as the soil exchangeable cations buffer changes in solution SAR. This effect is greater for soils with high cation exchange capacity.

Almost all studies of chemical effects on soil properties are based on short-term experiments with disturbed soil under continuous saturation. Suarez et al. (2006) demonstrated that the long-term effects on infiltration are more severe than current guidelines indicate. The revised water quality graph shown in Figure 3.2 (Suarez, 2010) represents pH dependent effects on infiltration as well as the EC and SAR interactions. It is emphasized that these are only guidelines and different responses may be expected for soils of varying stability.



**FIGURE 3.2** Sodicity hazard of irrigation water on water infiltration as related to EC, SAR, and pH. (After Suarez, D.L. Irrigation water quality assessments, in *Agricultural Salinity Assessment and Management*, ed. W. Wallender, Chap. 11, *Am. Soc. Civil. Eng.*, New York, 2010, in press.)

### 3.4 MANAGEMENT OPTIONS

#### 3.4.1 IMPROVED DELIVERY SYSTEMS

Ensuring that soils are not over-irrigated and maximizing the food production per unit of water applied requires changes in irrigation systems and management. Most improvements to date have been done on the engineering side, with relatively less change on the agronomic side. For example, conversion of surface flooding or furrow to sprinkler allows for more uniform application of water, reduced need for irrigation water, and reduced drainage volumes. Application of drip irrigation systems allows for uniform delivery of water to plants or trees in the field, while avoiding wetting of the entire soil surface. These system changes and associated changes in management practices require capital investments and education programs for irrigators. Nonetheless, these systems are less costly than development of new water supplies, especially the use of desalinated water.

In the instance of Grand Valley, Colorado, improved water delivery and management was essential for salinity control in the valley as well as for reduction in salinity in the lower regions of the Colorado River that receive the return flows. Improvements in irrigation system infrastructure and management in Grand Valley Colorado, including concrete lining of canals and laterals, installation of closed pipe delivery systems, and irrigation scheduling are calculated to have reduced the salt load to the Colorado River by approximately 500,000 ton per year.

#### 3.4.2 WATER REUSE

As discussed above, secondary salinization due to over-irrigation and insufficient drainage is the major cause of soil salinization in irrigated lands. Reuse of drainage water, where feasible, provides the opportunity for alternative water resources in water-short regions, as well as water-table control. A significant concern regarding the reuse of drainage water is its impact on the soil, and the potential salinization from applying more saline irrigation water on an already saline soil. However, Corwin et al. (1998) observed a decrease in soil salinity, and partial reclamation of sodic

soil conditions where drainage water more saline than presently formerly used irrigation water was applied. The benefits may be from several factors including a drop in the perched water table below the field, allowing for better drainage, as well as improved infiltration related to application of more saline water and application of greater volumes of water.

Maintaining irrigation in arid regions will require maximum utilization of sustainable water supplies. Water reuse is a necessary aspect of this system, but it should be looked at as a complimentary practice rather than as an alternative strategy for water management or as an alternative to reduction in drainage volumes. The ideal water use is still to extract the maximum benefit from the initial fresh water application, minimizing the volume of drainage water generated. This minimizes the need for drainage and avoids the mixing and degradation either of fresh water, if the drainage returns to a water supply such as a river, or else degradation of the drainage water by mixing with a saline ground water. This concept has been often dismissed as impractical. It has been argued that as crops vary in salt tolerance, application of water quantities at or near ET is feasible only for salt-tolerant crops if the irrigation water has any appreciable salinity. This argument is examined in the subsequent section.

### 3.4.3 REDUCTION IN QUANTITIES OF LEACHING WATER

The possibilities of using saline waters at low leaching fractions have been significantly overlooked due to use of current guidelines, such as Ayers and Westcot (1985). The major justification for application of water in excess of crop requirements has been the need to leach salts out of the root zone and thus control root-zone salinity. The leaching requirement concept provides for calculation of a crop-specific quantity of leaching water in addition to that consumed by the crop that must be applied to avoid yield loss to salinity.

The use of the static leaching requirement calculation is being questioned on several grounds. Most importantly, as demonstrated in an example below, the concept does not consider the decrease in water uptake and thus increase in leaching that occurs when plant yield decreases. The leaching fraction is thus not a fixed input variable but rather a result of water applications, potential ET, and plant response.

Secondly, the method used to calculate plant yield as related to salinity of irrigation water usually involves a simplified calculation of root-zone salinity, and the root-zone average value is used (Ayers and Westcot, 1985) rather than a water-uptake calculated value. The salinity in the deeper portions of the profile is greater than that near the surface, where the roots are concentrated and where most of the water is taken up by the plants. The calculation of average root-zone salinity thus overestimates the salinity experienced by the plant.

Most salt-tolerance data were, and are still, collected either in sand culture where the soil water salinity is essentially equal to the irrigation water salinity, or else at high leaching fractions where plant uptake weighted salinity is at most 50% greater than the irrigation water salinity. Also, the simplified calculations utilized do not account for the precipitation of calcite and possibly gypsum that occurs during the concentration of salts in the root zone, nor the nonlinearity between concentration increases and increases in EC and OP.

The combination of the assumption of fixed crop ET with the salt-tolerance calculation from average root-zone salinity estimates or measurements results in overestimation of the quantity of water needed for leaching. The lower the leaching fraction, the greater the discrepancy between average root-zone salinity and plant-uptake weighted salinity. This also explains why drip irrigation systems operated at or near the crop water requirement do not experience measurable yield losses, contrary to predictions based on the application of the leaching requirement concept.

Irrigation recommendations can best be made by using computer simulations of the dynamic processes, considering crop salt tolerance and crop ET and root-zone salinity based on predicted rather than potential water uptake. Letey and Feng (2007), comparing the results of a transient state model to those of a steady state model concluded that the transient model, consistent with field data, indicated that a much lower water application was required to avoid yield loss. Below, we compare the leaching requirements and prediction of yield loss between the SWS (Suarez et al., 2010) and

the Ayers and Westcot (1985) guideline recommendation for leaching and yield loss due to salinity. An analysis of the predicted ET, predicted leaching, and crop yield as related to irrigation water salinity is also presented below.

The user-friendly SWS version (Suarez and Vaughan, 2001; Suarez et al., 2010) of the UNSATCHEM model (Suarez and Simunek, 1997) predicts plant response to water and salt stress under dynamic conditions. The model also predicts soil solution composition as related to variably saturated water and solute transport and chemical processes of adsorption, mineral precipitation dissolution, and cation exchange. The model uses the predicted decreases in plant water uptake to predict the decrease in biomass production. This calculation assumes that yield is directly proportional to water consumption (constant WUE, or water use efficiency):

$$\frac{Y}{Y_M} = 1 - \beta_0 \left( 1 - \frac{ET_a}{ET_p} \right) \quad (3.7)$$

where

$Y$  is the actual yield

$Y_M$  is the maximum yield

$ET_a$  is the predicted or actual ET

$ET_p$  is the potential ET

The parameter  $\beta_0$  is a crop adjustable parameter, which is typically set to 1.0, but varies between 1.0 and 1.3 (Stewart et al., 1977).

Prediction of the yield of individual plant parts (such as seed or fruit) can be obtained by consideration of the relation of reduction in plant water uptake and yield response of the plant part of interest. The model predicted root-zone salinity and relative yield can be contrasted to predictions based on salt stress from guideline predictions.

In the following analysis, we use the SWS model (Suarez et al., 2010) to predict the plant yield reduction from salt stress. A perennial crop with a 100 cm root-zone depth on a loam soil ( $k_s = 25$  cm/d) was irrigated for 200 days. The first irrigation of 11 cm was applied after 10 days. After another 10 days, 22 cm of water was applied over 2 days followed by irrigations of 22 cm every 20 days thereafter for a total of 209 cm of applied irrigation water. The potential ET of the crop for full yield was 200 cm and we assumed a constant potential crop ET ( $ET_p$ ) value of 1 cm/d. The initial soil water and irrigation water composition were that of a predominately NaCl system with lesser quantities of Ca, Mg,  $SO_4$ , and bicarbonate. The  $h_{\phi 50}$  for osmotic stress was set at  $-50$  m, using the equation

$$\alpha_\phi(h_\phi) = \frac{1}{1 + \left( \frac{h_\phi}{h_{\phi 50}} \right)^p} \quad (3.8)$$

where

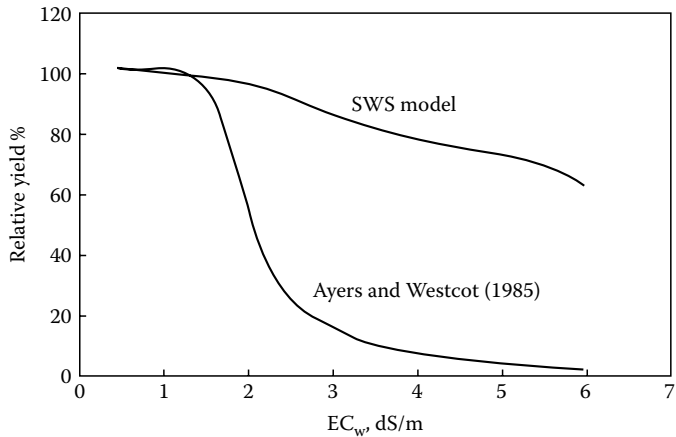
$\alpha_\phi$  is the osmotic stress response function (scaled from 0 to 1.0, where 1.0 equals no stress)

$h_\phi$  is the calculated osmotic stress

$h_{\phi 50}$  is the model input osmotic stress at which there is a 50% reduction in water use and relative yield

The same scenario was also evaluated using the Ayers and Westcot (1985) procedure. In this calculation, we consider the crop requirement of 200 cm of water and the applied water quantity of 209 cm. The average root-zone salinity was calculated from the average salinity of the root zone, using the irrigation water salinity and the salinity at the bottom of each of the four quarters of the root zone. The salinity in each quarter was based on the assumption that the water uptake is 40% in the first quarter, 30% in the second quarter, 20% in the third quarter, and 10% in the fourth quarter. The average



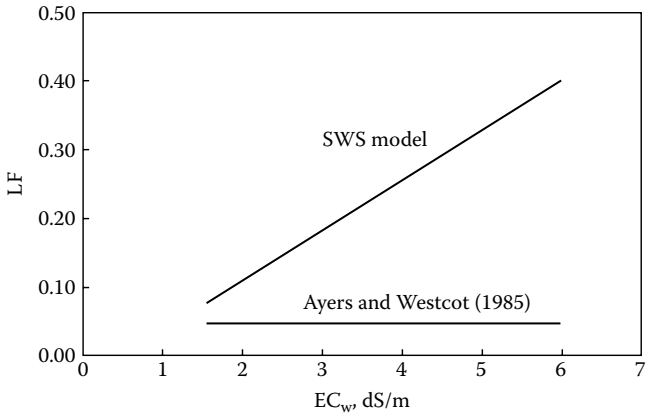


**FIGURE 3.3** Comparison of SWS model (Ayers and Westcot, 1985; Suarez et al., 2010) predicted crop relative yield as related to irrigation water EC, for a crop with an  $h_{50} = -50$  m ( $-0.5$  MPa),  $ET_p = 200$  cm and 209 cm applied water.

root-zone salinity was thus calculated and converted to OP using the conversion factor  $OP$  (MPa) =  $-0.4$   $EC$  (dS/m), and using Equation 3.8 the stress factor and relative yield was obtained.

The SWS model predicted relative yield as related to irrigation water salinity is shown in Figure 3.3. The model predicts a gradual decline in relative yield with increasing irrigation water salinity. With an irrigation water EC of 4.0 dS/m the relative yield is still at 81%, despite the application of only a small amount of water above the crop potential ET. In contrast, as shown in Figure 3.3, the Ayers and Westcot (1985) calculated yield decreases rapidly above EC 1.5 dS/m. We conclude from the guideline calculations that, for this salt-tolerance data ( $h_{50} = -05$  MPa), irrigation with water above  $EC = 2.0$  dS/m is not feasible for efficient irrigation practices at a leaching fraction of 0.05. As seen in Figure 3.3, at higher irrigation water salinities, there is a dramatic difference between the model and guideline prediction. A similar result to that obtained by calculation from the FAO guidelines (Ayers and Westcot, 1985) would also be obtained using the steady state WATSUIT model (Rhoades and Merrill, 1976).

As shown in Figure 3.4, the guideline assumes constant water consumption even as yield approaches zero. The model predictions show that the decrease in plant water uptake is associated



**FIGURE 3.4** Comparison of SWS model (Ayers and Westcot, 1985; Suarez et al., 2010) predicted leaching fraction as related to irrigation water EC, for a crop with an  $h_{50} = -50$  m ( $-0.5$  MPa) salt-tolerance value,  $ET_p = 200$  cm and 209 cm applied water.

with salt stress, thus, increased leaching with increased salinity of irrigation water. The reduction in water uptake moderates the increase in root-zone salinity. Consideration of the actual water budget is essential for the calculation of the actual salinity in the root zone. The increased leaching and decreased water uptake was due entirely to salt (osmotic stress).

The major discrepancy between these calculations and the SWS predictions is the failure of these calculations to predict the reduction in water consumption by the crop and thus the root-zone salinity and leaching fraction. The leaching fraction was assumed to be 0.043, based on the applied water and crop water demands (ET); however, the SWS model predicted reduced water uptake and a LF = 0.42 at the highest irrigation water salinity examined. The differences between the model predictions (less stress) and the simple calculation method are even greater when we consider waters that precipitate gypsum in the soil, thus reducing the salt concentrations in the soil.

While the above example is somewhat extreme in terms of the close correspondence between water application and crop water demand for full yield (209 cm vs. 200 cm), such irrigation efficiency is not unusual for new irrigation technologies, such as drip irrigation. It appears that dynamic modeling is necessary for irrigation management when low-target leaching fractions are the objective under conditions of potential yield loss due to salinity.

As observed by data collected from drip systems, water applications can be greatly reduced and still maintain yield in most environments. This in turn suggests that less drainage water of higher salinity will be generated, thus disposal for maintaining ground water levels will be reduced.

### 3.4.4 CROP QUALITY AND ECONOMIC CONSIDERATIONS

The classification and consideration of the suitability of saline and brackish waters for irrigation have focused on the threshold of salt-tolerance levels and leaching necessary for full maximum production. As indicated earlier, the leaching needs of current guidelines are excessive. Equally important, such calculations do not consider the farmers objective to optimum profit and the societal need for optimum use of resources. Profitability and societal needs for local food production may make even large decreases in relative yield still feasible, especially when alternative water supplies do not exist. These economic considerations should be inputs to the decisions regarding water use, crop selection, and acceptable yields. Selecting more salt-tolerant crops that do not have projected yield losses may also not be optimal. For example, tall wheatgrass is more salt tolerant than alfalfa; however, alfalfa outyielded tall wheatgrass in controlled studies at EC soil water of 15 dS/m (Grattan et al., 2004).

In some instances, the adverse impacts of reduced yields may be compounded by reduced crop quality such as smaller fruit size, thus decreasing marketability. However, in some instances, crop quality may improve under saline conditions; at least partially offsetting yield reductions. Recently, Grieve (2010) examined the characteristics or composition variables that were improved by salinity for a variety of crops. These benefits include increased sugar content of many crops, including tomato, carrots, onions, and melons, among others. Salt stress may also increase antioxidants and improve fruit flavor and firmness (Grieve, 2010).

### 3.4.5 POTENTIAL FOR INCREASED SALT TOLERANCE

Biotechnology in combination with conventional breeding practices holds great promise to improve salt tolerance, especially of crops that are sensitive or moderately sensitive to salinity. It is generally assumed that the adverse response of plants to elevated concentrations of salt is due to the increased OP of the soil water. The plant is considered to divert energy into extracting low salinity water from the more saline soil water, thus impacting plant growth. However, there is a very wide range in salt tolerance, starting at very low salinity levels such as less than 1.0 dS/m for strawberry. There is strong evidence that specific ion toxicity is the major impact on salt sensitive species.

Munns and Tester (2008) considered that plant response to salinity could be represented by a two part process, with the initial adverse response being related to increased OP and a later response related to specific ion toxicity. They consider that the toxic ion effect dominates for salt sensitive species that lack the ability to control  $\text{Na}^+$  transport, and that for all other plant species the ionic effect is important only at high salinity. Development of salt-tolerant varieties of sensitive species can thus be accomplished by focusing on the development of improved  $\text{Na}^+$  (and to a lesser extent  $\text{Cl}^-$ ) exclusion by the roots and restriction of translocation to the leaves. Additionally, tissue tolerance to salinity by plants is achieved by compartmentalization of  $\text{Na}^+$  and  $\text{Cl}^-$  at the cellular and intracellular level.

### 3.5 CONCLUSIONS

There is very limited potential for using fresh water for increased development of irrigation in arid regions. More realistically, there will be a significant decrease in fresh water use, due to current unsustainable extractions of fresh water. More efficient use of available resources includes use of new irrigation technologies, reuse of drainage water, use of treated municipal waste water, use of brackish water, and reduced leaching for salinity control. However, especially in the presence of rain, the sodicity hazard of lower quality irrigation waters is of concern, since commonly used water quality guidelines may not be sufficiently protective to maintain adequate infiltration. Replacement of current simplified guidelines for leaching with more realistic computer models indicates a decreased need for leaching and will enable better salinity management and use of resources. Opportunities also exist for the development of improved salt tolerance for varieties of salt sensitive plant species.

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# 4 Influence of Sodium on Soils in Humid Regions

*Rafif K. Srour, Louis M. McDonald, V.P. (Bill) Evangelou<sup>†</sup>*

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## 4.1 INTRODUCTION

There is a great deal of information on the behavior of salts in soils and in soil solution suspensions. However, most of this research applies to soils in arid regions, which are often alkaline and consist primarily of 2:1 clay minerals. In temperate regions, soils are often highly weathered, acidic, their mineralogy oxidic, with mixed clay mineralogy (1:1 plus 2:1 clay minerals), and the 2:1 minerals are highly interlayered. There is a need to understand the effects of salts on soils in temperate regions because these soils can become salt-affected from anthropogenic sources. This information is needed by landowners and managers and by state and federal regulatory agencies to protect vital resources.

A salt-affected soil is defined as one that has been adversely affected by the presence or action of soluble salts to the extent that it is no longer suitable for the growth of most crops. This group of soils includes both saline and sodic soils. A saline soil is one that contains an excess of soluble salts, typically  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ , and  $\text{Na}^+$  and  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ , and  $\text{Cl}^-$ . A sodic soil possesses an excess of exchangeable sodium. A saline–sodic soil contains both soluble salts and exchangeable Na at levels that impose stress on plant growth (Table 4.1).

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<sup>†</sup> Deceased.

**TABLE 4.1**  
**Standard U.S. Definitions of Salt-Affected Soils**

| Name         | Saturated Extract<br>Conductivity<br>(EC <sub>e</sub> ) (dS m <sup>-1</sup> ) | Sodium Adsorption<br>Ratio (SAR)<br>(mmol L <sup>-1</sup> ) <sup>0.5</sup> |
|--------------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| Unaffected   | <4                                                                            | <13                                                                        |
| Saline       | ≥4                                                                            | <13                                                                        |
| Sodic        | <4                                                                            | ≥13                                                                        |
| Saline-sodic | ≥4                                                                            | ≥13                                                                        |

Source: Courtesy of Soil Science Society of America,  
Madison, WI.

Salt-affected soils are a common feature of arid and semiarid landscapes because evapotranspiration (ET) exceeds precipitation (P). In humid regions, where  $P > ET$ , soluble salts are leached, but soils may become salt-affected when they are irrigated with brackish water (Kaur et al., 2008) or treated sewage effluent (Leal et al., 2009), intruded by sea water, excessively fertilized with manures (Kuo, 1981; Murphy et al., 2005), or contaminated with oil and gas well brines. Compared to arid and semi-arid regions, detailed studies of salt-affected soils in humid and tropical climate zones are, so far, quite limited (Wongpokhom et al., 2008).

The loss of plant productivity from excess salinity is a worldwide problem. Where salinity is a problem, the effective use and management of salt and water resources dictates the production of agricultural crops. Numerous laboratory and field experiments have been conducted in order to determine the plant growth and yield response to various levels of soil salinity. For example, Shalhevet et al. (1969) found that the yield of peanuts grown in artificially salinized plots was reduced to 50% at EC<sub>e</sub> (specific electrical conductance (EC) of a saturated extract [e]) of 4.7 dS m<sup>-1</sup> and by 20% at EC<sub>e</sub> of 3.8 dS m<sup>-1</sup>. Additionally, these investigators reported that salt tolerance was much higher during germination than during subsequent growth; a comparable 50% reduction in germination did not occur until an EC<sub>e</sub> of 13 dS m<sup>-1</sup>. Shalhevet and Yaron (1973) reported a 10% yield reduction in tomato for every 1.5 dS m<sup>-1</sup> increase in EC<sub>e</sub> above 2 dS m<sup>-1</sup>. The adverse effects of soil salinity on plant growth and productivity vary with the type of plant being grown as well as the type of soil and climate considered. In semi-humid regions of the North China Plain, few effects on tomato plant yield were observed when the irrigation water salinity was increased from 1.1 to 4.9 dS m<sup>-1</sup>. However, higher salinity improved both tomato water use efficiency and irrigation water use efficiency (Wan et al., 2007).

A few studies have examined the effects of soil salinity/sodicity on soil quality as measured by biological processes such as on soil microbial biomass (SMB) and microbial activity (e.g., Sarig et al., 1993; Pathak et al., 1998; Tripathi et al., 2006; Wong et al., 2008). In general, low salinity soils have higher soil respiration rates and lower SMB (Wong et al., 2008). However, available studies often show contradictory results (e.g., Chander et al., 1994; Nelson et al., 1996; Rietz and Haynes, 2003), and in highly saline soils (30 dS m<sup>-1</sup>), the high salt concentration caused elevated SMB as a result of increased substrate availability (Wong et al., 2008). The apparent disparity in trends in respiration and the SMB reported in the literature may be caused by the shifts in the microbial population (from a more active population to a less active one) caused by salinity and/or sodicity (Wong et al., 2008).

Salinity in the soil solution resulting from either indigenous or anthropogenic salt can affect plant growth in three ways (James et al., 1982). It can increase the osmotic potential thus decreasing water potential, thereby reducing water availability, the *osmotic effect*. It can increase the concentration of ions that have an inhibitory effect on plant metabolism, the *specific ion effect*

(James et al., 1982). It can adversely affect soil structure such that water permeability and soil aeration are diminished, the *physiochemical effect*. In many cases, these effects are not mutually exclusive and often overlap in complicated ways.

#### 4.1.1 OSMOTIC EFFECT

Under normal field conditions, the soil–water potential ( $U_w$ ) is determined by the osmotic potential ( $U_s$ ), the matrix potential ( $U_m$ ), and the gravitational potential ( $U_p$ ), such that

$$U_w = U_m + U_s + U_p \quad (4.1)$$

At any given matrix potential and a fixed gravitational potential, an increase in salinity causes a decrease in  $U_w$  (James et al., 1982). Based on the principle that the amount of EC transmitted by a salt solution increases as salt concentration increases. The U.S. Salinity Laboratory Staff (1954) described the relationship as a function of EC,

$$U_s \text{ (bar)} = -0.36 \times \text{EC (dS m}^{-1}\text{)} \quad (4.2)$$

Because the  $U_s$  of a solution is directly related to total dissolved solids (TDS) concentration (Bresler et al., 1982), the relationship can also be expressed as

$$U_s \text{ (bar)} = -5.6 \times 10^{-4} \times \text{TDS (ppm)} \quad (4.3)$$

#### 4.1.2 SPECIFIC ION EFFECT

An excess of Na ions in the soil solution can be inhibitory to plant physiological processes. The sensitivity of plants to Na levels in the soil solution and/or soil surfaces is highly dependent on plant species, as well as plant development stage (Hausenbuiller, 1978; Donahue et al., 1983). Symptoms of Na toxicity can be easily seen when the leaves of sensitive plants contain approximately 0.25% Na on a dry-weight basis (Bresler et al., 1982; James et al., 1982). Sodium toxicity is characterized by leaf tip burn, necrotic spots, and limited leaf expansion, which in turn directly reduces plant photosynthesis and yield (Bernstein, 1975; Neumann et al., 1988).

Other  $\text{Na}^+$  specific ion effects on plant physiological processes are observed when plants are grown in high-Na environments. High sodium concentrations have been shown to increase  $\text{K}^+$  leakage and decrease root elongation in mung bean. Nakamura et al. (1990) reported that when  $\text{Na}^+$  is present in high concentration in the solution, it reduces the transpiration rate of peas (Meiri and Poljakoff-Mayber, 1970) and negatively affects the respiratory pathway of pea roots (Porath and Poljakoff-Mayber, 1964). High  $\text{Na}^+$  concentrations in soil solution also has an antagonistic effect on  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  uptake, causing Ca deficiency symptoms in tomato, pepper, and celery plants (Geraldson, 1957). This was most likely caused by  $\text{Na}^+$  displacing  $\text{Ca}^{2+}$  from membranes of root cells.

#### 4.1.3 PHYSICOCHEMICAL EFFECT

While salinity can be directly detrimental to crop and microorganism growth, it promotes aggregation of soil particles, resulting in structurally stable soils with enhanced permeability and hydraulic conductivity and low susceptibility to shrinking, swelling, and cracking (Mitchell and van Genuchten, 1992; Buckland et al., 2002; Bauder et al., 2008). However, an excess of exchangeable Na, as is the case in sodic soils, is indirectly harmful to plants because it increases clay dispersion, reduces permeability, and causes soil structure to deteriorate.

When soils are negatively charged, the composition of the soil surface (exchanger) is related to the composition of the soil solution. For salt-affected soils, these relationships are described by the exchangeable sodium percentage (ESP) and the sodium adsorption ratio (SAR), such that

$$\text{ESP} = 100 \times \frac{\text{Exchangeable sodium content (cmol}_c \text{ kg}^{-1})}{\text{Cation exchange capacity (cmol}_c \text{ kg}^{-1})} \quad (4.4)$$

and

$$\text{SAR (mmol}_c \text{ L}^{-1})^{0.5} = \frac{[\text{Na}^+]}{[\text{Mg}^{2+} + \text{Ca}^{2+}]^{0.5}} \quad (4.5)$$

where brackets indicates total ion concentration in  $\text{mmol L}^{-1}$ . An empirical relationship between ESP and SAR for soils of the arid Western United States was developed by the U.S. Salinity Laboratory Staff (1954) as

$$\text{ESP} = \frac{100(-0.0126 + 0.014575 \cdot \text{SAR})}{1 + (-0.0126 + 0.014575 \cdot \text{SAR})} \quad (4.6)$$

When SAR is approximately in the range of 10–15, ESP is also in the range of 10–15. In this ESP range, soils of the arid West undergo dispersion and plant growth suffers. However, this relationship does not apply to all soils. For example, a SAR value of 5 was considered to define sodicity for Australian soils (Rengasamy and Olsson, 1991). ESP–SAR relationships can also vary within a soil profile. Mohamed et al. (2008) suggested different regression equations for ESP and SAR for different horizons. Their analyses of more than 90 samples collected in soil profiles from various climatic zones ranging from arid to tropical, revealed that ESP was more variable than SAR in A- and AC-horizons but less variable in C-horizons. However, for simplicity, they proposed a different quadratic equation only for saline and sodic soils. For saline soils,

$$\text{ESP} = 2.855 + 0.73 \cdot \text{SAR} + 0.008 \cdot \text{SAR}^2 \quad R^2 = 0.96 \quad (4.7)$$

and for sodic soils,

$$\text{ESP} = -7.49 + 1.34 \cdot \text{SAR} - 0.0056 \cdot \text{SAR}^2 \quad R^2 = 0.75 \quad (4.8)$$

## 4.2 THERMODYNAMICS OF SODIUM–CALCIUM EXCHANGE IN SOILS

Soils are multicomponent systems consisting of solid (inorganic and organic components), liquid (soil solution), and gaseous phases. These three dynamic phases are to some extent in a constant state of flux, trying to maintain a state of equilibrium. A change in one phase will influence the other two phases until a new equilibrium state is reached. Cation exchange is one type of equilibrium interaction. This involves interchange between cations in the solid phase with other cations in the solution phase.

Cation exchange reactions result from the excess negative charge on soil colloids. There are two types of negative charge in soil systems (Gast, 1977; White and Zelazny, 1986). A permanent negative charge is generated because of isomorphic substitution of elements of smaller positive charge for those of higher positive charge in the crystal structure of clay minerals. Variable negative charge on mineral surfaces and organic matter results from the protonation and deprotonation of surface functional groups (Talibudeen, 1981). The magnitude of the variable negative charge is influenced by pH as well as ionic strength. An increase in pH and/or ionic strength is followed by an increase in negative charge

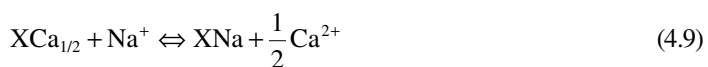


(Gillman, 1984). In soil systems of temperate regions, these two types of negative charges are always present, but in some soils, one type of negative charge is more dominant than the other.

Because soils contain a mixture of various types of clay minerals and because more than two cations are present in such soil systems (Ca, Mg, K, Na,  $\text{NH}_4$ ), a rigorous theoretical description of ionic distribution is difficult. Several theoretical approaches have been used in deriving binary exchange equations; for salt-affected soils these have been for sodium–calcium exchange. Those most often mentioned in the literature are the thermodynamic and the double layer approaches. The formal thermodynamic approach, based on the mass action principle, gives no direct information about the molecular mechanisms and the forces operating in such systems. On the other hand, the diffuse double layer approach provides a description of Coulombic forces operating on ion exchange processes (Shainberg and Letey, 1984).

#### 4.2.1 SODIUM–CALCIUM EXCHANGE THEORY: MASS ACTION

Various attempts have been used to characterize the relationship between ESP and SAR. The mass action approach is based on the equilibrium established between the chemical potentials of the soil solution and the exchanging surface. The two most common conventions for defining exchanging surface chemical potentials are the Gapon and the Vanselow conventions, with the Gapon convention being more frequently used to characterize salt-affected soils. In the Gapon convention, for the binary chemical reaction



where

X is a cation exchanger taken to have a charge of negative one (–1)

$\text{Na}^+$  and  $\text{Ca}^{2+}$  denote solution species

The Gapon exchange selectivity coefficient is defined as

$$K_G = \frac{[\text{XNa}]}{[\text{XCa}_{1/2}]} \times \frac{[\text{Ca}^{2+}]^{1/2}}{[\text{Na}^+]} \quad (4.10)$$

where

$[\text{Ca}^{2+}]$  and  $[\text{Na}^+]$  are expressed in  $\text{mmol L}^{-1}$

$[\text{CaX}]$  and  $[\text{Na}_2\text{X}]$  are expressed in units of cation exchange capacity (CEC),  $\text{cmol}_c \text{ kg}^{-1}$  or  $\text{meq (100 g)}^{-1}$

Because Ca and Mg behave similarly in soils, the presence of Mg can easily be accounted for as

$$K_G = \frac{[\text{XNa}]}{[\text{X(Ca + Mg)}_{1/2}]} \times \frac{[\text{Ca}^{2+} + \text{Mg}^{2+}]^{1/2}}{[\text{Na}^+]} \quad (4.11)$$

where the exchangeable sodium ratio (ESR) is

$$\frac{[\text{XNa}]}{[\text{X(Ca + Mg)}_{1/2}]}, \text{ or } \frac{[\text{XNa}]}{[\text{XCa}_{1/2}]} \quad (4.12)$$

the ESP is

$$\text{ESP} = 100 \times \frac{[\text{XNa}]}{\text{CEC}} \quad (4.13)$$

and the SAR is

$$\frac{[\text{Na}^+]}{[(\text{Ca} + \text{Mg})]^{1/2}}, \quad \text{or} \quad \frac{[\text{Na}^+]}{[\text{Ca}^{2+}]^{1/2}} \quad (4.14)$$

The Gapon convention has been criticized for lacking thermodynamic rigor and so most quantitative studies of cation exchange phenomenon use the Vanselow convention. Both conventions have been reviewed elsewhere (e.g., Evangelou and McDonald, 1999; Evangelou and Phillips, 2005; McDonald, et al., 2005), as have other conventions (e.g., Essington, 2004).

Bohn et al. (1985) summarized the limitations of most cation exchange equations as follows: (a) Binary cation exchange is frequently considered but rarely the simultaneous presence of additional cations is acknowledged even for highly acidic systems. (b) The cation exchanger is assumed to possess constant cation exchange capacity, but often cation exchange capacity varies with the nature of the exchanging ions, solution concentration, and pH. (c) Simple stoichiometric (1:1) ion exchange is generally assumed, but the apparent deviations from 1:1 stoichiometry are usually explained in terms of simultaneous adsorption of molecules or in terms of the formation of complex ions. (d) Complete reversibility is usually taken for granted.

#### 4.2.2 SODIUM–CALCIUM EXCHANGE THEORY: DIFFUSE DOUBLE LAYER

In the diffuse double layer theory, the fraction of the surface charge neutralized by monovalent ions ( $\sigma_1/\sigma_0$ ) is proportional to SAR and was given as (Erickson, 1952; Bolt, 1955a)

$$\frac{\sigma_1}{\sigma_0} = \frac{\text{SAR}}{31.6\sigma_0\sqrt{\beta}} \sinh^{-1} \frac{31.6\sigma_0\sqrt{\beta}}{\text{SAR} + 126.4u_d\sqrt{\text{Ca}}} \quad (4.15)$$

where

$$\text{SAR} = \frac{n_1^\infty}{\sqrt{n_2^\infty}} \sqrt{1000} \quad (4.16)$$

and

$$\beta = \sqrt{\frac{4e^2}{\epsilon kT}} \quad (4.17)$$

and

$$u_d = \cosh \frac{e\phi_d}{kT} \quad (4.18)$$

where

$e$  is the electronic charge

$\phi$  is the electrical potential as a function of distance ( $d$ ) from the surface

$k$  is Boltzmann's constant

$\epsilon$  is the dielectric constant

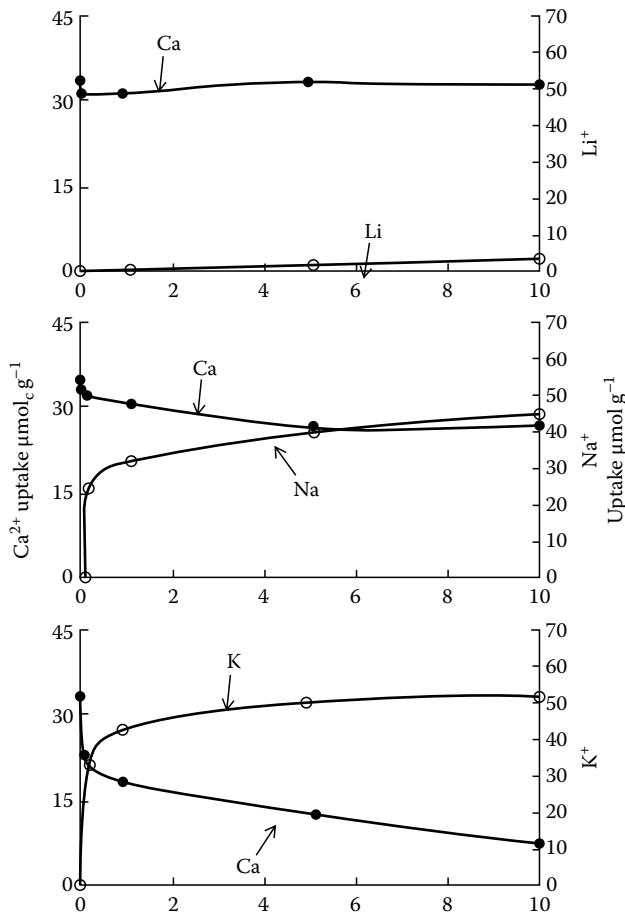
$T$  is temperature

$n_1^\infty = \text{Na}$ , and  $n_2^\infty = \text{Ca}$

From Equations 4.15 and 4.16 and recognizing the shape of the function  $y = \sinh^{-1}(x)$ , the following observations can be made. First, increasing SAR increases  $\sigma_1/\sigma_0$ , although not linearly. Second,

increasing ionic strength, which is accounted for by the  $\sqrt{Ca}$  term, decreases  $\sigma_1/\sigma_0$  (Babcock, 1963). And third, increasing CEC ( $-\sigma_0$ ) increases  $\sigma_1$  but decreases  $\sigma_1/\sigma_0$  (Babcock, 1963). All of these observations are consistent with the results of Evangelou and Phillips (1987). Typically it has been assumed that the soil particles were sufficiently far apart so that  $\phi_d = 0$  and  $u_d = 1$  (Bolt, 1955b; Bower, 1959). Shainberg et al. (1980) have shown that in systems where tactoids are formed such that  $\phi_d \neq 0$ , increasing ionic strength increases  $\sigma_1$  for the internal tactoid surfaces but decrease  $\sigma_1$  for the external clay surfaces. This suggests that montmorillonitic soils will behave differently than soils with mixed mineralogy (Arora and Coleman, 1979; Shainberg et al., 1980).

The above equations have implications for ion uptake by plants as well as ion exchange reactions on soil surfaces. Because cell membranes are negatively charged, the presence of an aqueous solution establishes an electric double layer. In many, but not all cases, ion toxicity effects are more closely correlated with ion (concentrations) activities at the membrane surface than with bulk solution ion (concentrations) activities (Kinraide, 1994). In these instances, it is possible to rationalize the effects of ion interactions on ion uptake without invoking the presence of specific ion carriers, multiple sites or other metabolic explanations. For example, Maas (1969) evaluated the effect of increasing concentrations of Li, Na, and K on the uptake of Li, Na, K, and Ca into excised maize roots (Figure 4.1). The results (Figure 4.1) are entirely consistent with the above equations. In Equation 4.16, increasing  $r$  (the relative monovalent ion concentration) increases  $\sigma_1/\sigma_0$ , and hence



**FIGURE 4.1** The effect of increasing  $K^+$ ,  $Na^+$ , and  $Li^+$  concentration on the uptake of  $Ca^{2+}$ ,  $K^+$ ,  $Na^+$ , and  $Li^+$  in 24 h. The concentration of  $Ca^{2+}$  was  $10 \text{ mmol}_c \text{ L}^{-1}$  and the pH was 6. (Redrawn from Mass, E.V., *Plant Physiol.*, 44, 985, 1969. With permission.)

the monovalent ion concentration in the double layer. Accepting that for an ion to move into a cell it must first move to the surface of the cell membrane, increasing the relative monovalent ion concentration in the solution phase necessarily increases ion uptake. When comparing Li, Na, and K, the extent to which ion uptake increases should be proportional to the ability of the ion to move into the Stern layer. That is, increasing the solution phase Li concentration has a modest effect on Li uptake, whereas, increasing the solution K concentration has a large effect on K uptake. The effects of monovalent ion concentration on Ca uptake can be explained similarly. To suppress Ca uptake, the monovalent ion has to compete at the Stern Layer level. Therefore, from Shainberg and Kemper's (1966) analysis one would predict that K would be more effective at suppressing Ca uptake than Li.

### 4.3 EFFECTS OF SOLUTION PROPERTIES ON SODIUM–CALCIUM EXCHANGE

Because single ion activity coefficients are used to quantify ion exchange equilibria (Equations 4.10 and 4.14), any property of a solution that affects these coefficients will affect the resulting equilibria. The single ion activity,  $\{i\}$ , describes the behavior of a particular chemical species in solution as

$$\{i\} = \gamma_i c_i \quad (4.19)$$

where

$\gamma_i$  denotes the single ion activity coefficient

$c_i$  the molar concentration of the chemical species

In ideal dilute aqueous solutions,  $\gamma_i$  equals 1 and the ionic activity equals the molar concentration. In aqueous solutions and in complex nonaqueous solutions,  $\gamma_i$  decreases, as predicted by the Debye–Hückel equation

$$\log \gamma_i = -\frac{Az_i^2 I^{0.5}}{1 + Ba_i I^{0.5}} \quad (4.20)$$

where

$z_i$  is the ionic charge

$a_i$  is the ion size parameter

$I$  is ionic strength

$A$  and  $B$  are two parameters related to the solvent properties and the temperature of the solution

$$A = (2\pi N_A \rho)^{0.5} \left[ \frac{e^2}{4\pi \epsilon \epsilon_0 kT} \right]^{1.5} \quad (4.21)$$

$$B = e_0 \left( \frac{2N_A \rho}{\epsilon \epsilon_0 kT} \right)^{0.5} \quad (4.22)$$

where

$N_A$  is Avogadro's number

$\rho$  is the solvent bulk density

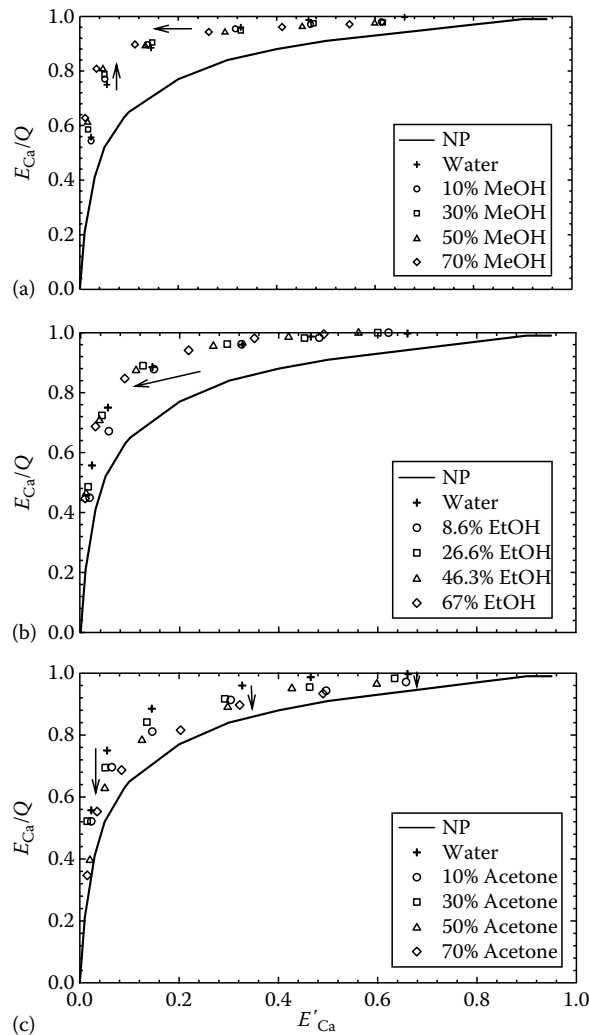
$\epsilon$  is the solvent dielectric constant

$\epsilon_0$  is the permittivity of vacuum

If all variables are kept constant, Equations 4.20 through 4.22 predict that a decrease in  $\epsilon$  would decrease the single ion activity coefficient and, hence, reduces its activity (Equation 4.19). Increasing  $I$  decreases  $\gamma$  for divalent ions more than for monovalent ions.

### 4.3.1 DIELECTRIC CONSTANT EFFECTS

The effects of changing dielectric constant of a solution are demonstrated for calcium–sodium exchange on Wyoming bentonite in methanol, ethanol, and acetone–water systems at 0.03 N Cl and at room temperature (Srou and McDonald, 2005). Calcium–sodium exchange isotherms were plotted at cosolvent concentrations ranging from 0%–70% wt/wt using Ca and Na ionic activities (Figure 4.2). A greater selectivity of bentonite surfaces for Ca ions was observed in all treatments. When compared to water, different trends were observed among and within cosolvents. In methanol–water systems, the preference of bentonite for Ca increased with increased methanol percent at low equivalent Ca fraction (<0.2). At higher Ca fractions, this preference matched that of water. In ethanol–water, no increased preference of the surface for Ca was observed. In acetone–water, increasing cosolvent concentration decreased the preference of the surface for Ca (Figure 4.2). The magnitude of this decrease was greater at low equivalent Ca fraction (<0.2). It is important to note that these isotherms were plotted without



**FIGURE 4.2** Ca–Na exchange isotherms on Wyoming Bentonite at 0.03 N Cl in methanol–water (a), ethanol–water (b) and acetone–water systems (c). (Arrows indicate changes in position of individual data points within and between isotherms.) NP represents nonpreference isotherms in water.  $E_{Ca}$  and  $E'_{Ca}$  are the equivalent fraction of Ca on the exchanger site and in solution, respectively.  $Q$  is the total adsorbed metal charge in  $\text{mol}_e \text{ kg}^{-1}$ . (Reproduced from Srou, R.K. and McDonald, L., *Clays Clay Mineral*, 53(5), 536, 2005.)

accounting for ion pair formation, mainly  $\text{CaCl}^+$ . After correction for  $\text{CaCl}^+$ , both in solution and on the surface (data not shown), the preference of bentonite for  $\text{Ca}^{2+}$  was larger in methanol- and ethanol-water systems. In acetone-water, increased surface preference for Ca was only apparent at low acetone fractions (<50%). At higher acetone fractions, there was evidence of increased Na-loading but no increase in  $\text{Ca}^{2+}$  selectivity.

4.3.2 IONIC STRENGTH AND pH

A large number of studies involving Na–Ca exchange have been conducted with respect to influence of ionic strength and solution composition. Few studies, however, have dealt with the role of pH on Na–Ca exchange reactions. This omission could be due to the fact that most salt-affected soils in the arid and semi-arid West exhibit pH values in the neutral range. Salt-affected soils in temperate regions of the United States are often acid in nature and of mixed type of charge site mineralogy. That is, they are composed of minerals that contribute significant quantities of variable charge.

In order to demonstrate the influence of salt concentration (ionic strength) and soil pH on  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange on soils representative of humid regions, data on two such soils are given here. These two soils are the Pembroke (fine silty, mixed, mesic, Mollic Paleudalf) from Hardin County, Kentucky, and the Uniontown (fine silty, mixed, mesic, Typic Hapludalf) from Union County, Kentucky (Marsi and Evangelou, 1991a, I). The Pembroke soil is much higher in clay content than the Uniontown soil. The Pembroke soil is dominated by kaolinite and to a lesser extent by mica, vermiculite, and hydroxy-interlayered vermiculite and smectite. The Uniontown soil is dominated by vermiculite and to a lesser extent by mica, kaolinite, and hydroxy-interlayered vermiculite and smectite. Another important difference is the much greater iron content of the Pembroke soil.

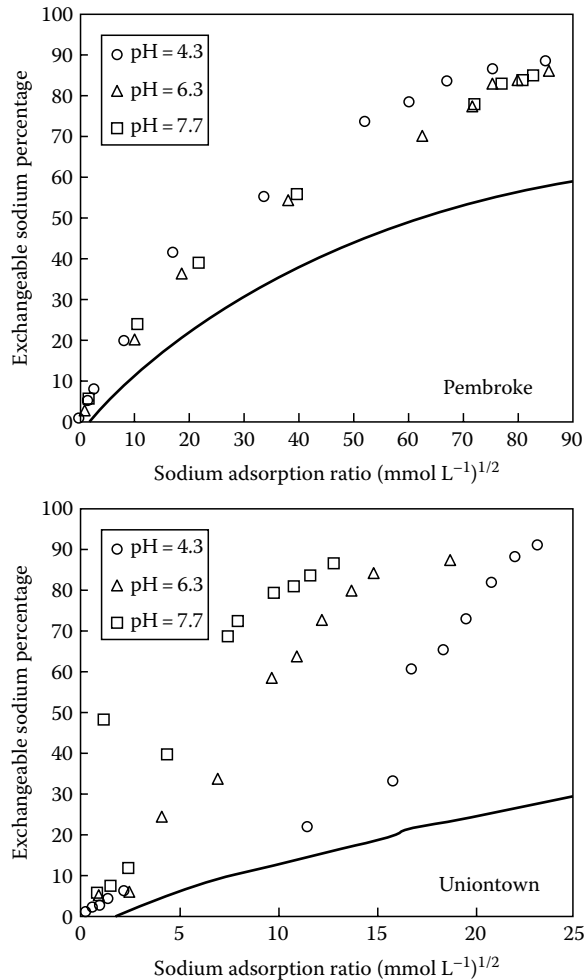
Data in Table 4.2 show the mean value of the summation of exchangeable  $\text{Na}^+$ , ExNa, and exchangeable  $\text{Ca}^{2+}$ , ExCa as a function of pH and chloride concentration for the Pembroke and the Uniontown soils, respectively. Each mean value reported is represented by 15 different ExNa or ExCa loads. The plus or minus value associated with each mean value represents the difference in metal adsorption when one of the metals ( $\text{Na}^+$  or  $\text{Ca}^{2+}$ ) on the exchanger phase approaches zero. Thus for any mean value plus the deviation from the mean, the sum signifies the effective charge ( $\text{EC}_g$ ) of the soil when the latter is loaded with  $\text{Ca}^{2+}$ , and for any mean value minus the deviation from the mean, the difference signifies the  $\text{EC}_g$  of the soil when the latter is loaded with  $\text{Na}^+$ . The data in Table 4.2 clearly demonstrates that the  $\text{EC}_g$  of these two soils is highly ionic strength dependent, specific ion dependent, and to a lesser extent pH dependent. The variation in effective soil charge as a function of the type of metal was previously reported by Fletcher et al. (1984), Hutcheon (1966), and Faucher and Thomas (1954). Murtaza et al. (2001) reported a decrease in the rate of Ca–Na

TABLE 4.2  
Mean – Sum *M* of Exchangeable Na and Ca of Pembroke and Uniontown  
Soils as a Function of pH and Chloride Concentration<sup>a</sup>

| Cl (mmol L <sup>-1</sup> ) | Pembroke (cmol <sub>c</sub> kg <sup>-1</sup> ) |            |            | Uniontown (cmol <sub>c</sub> kg <sup>-1</sup> ) |            |            |
|----------------------------|------------------------------------------------|------------|------------|-------------------------------------------------|------------|------------|
|                            | pH 4.3                                         | pH 6.1     | pH 7.5     | pH 4.3                                          | pH 6.3     | pH 7.7     |
| 5                          | 7.4 ± 0.2                                      | 8.8 ± 0.3  | 9.7 ± 0.4  | 8.9 ± 0.4                                       | 9.5 ± 0.5  | 10.5 ± 0.6 |
| 50                         | 11.2 ± 1.4                                     | 12.7 ± 1.3 | 13.6 ± 1.2 | 11.2 ± 1.4                                      | 13.4 ± 1.6 | 14.1 ± 1.5 |
| 200                        | 27.7 ± 3.3                                     | 27.7 ± 5.5 | 28.9 ± 5.4 | 20.8 ± 2.1                                      | 23.2 ± 2.8 | 25.4 ± 2.9 |

Source: Marsi, M. and Evangelou, V.P., *Environ. Sci. Health*, A26(7), 1147, 1991b. With permission.

<sup>a</sup> M + S = effective charge ( $\text{EC}_g$ ) of Ca-loaded soil; M – S = effective charge ( $\text{EC}_g$ ) of Na-loaded soil; S = deviation from the average.

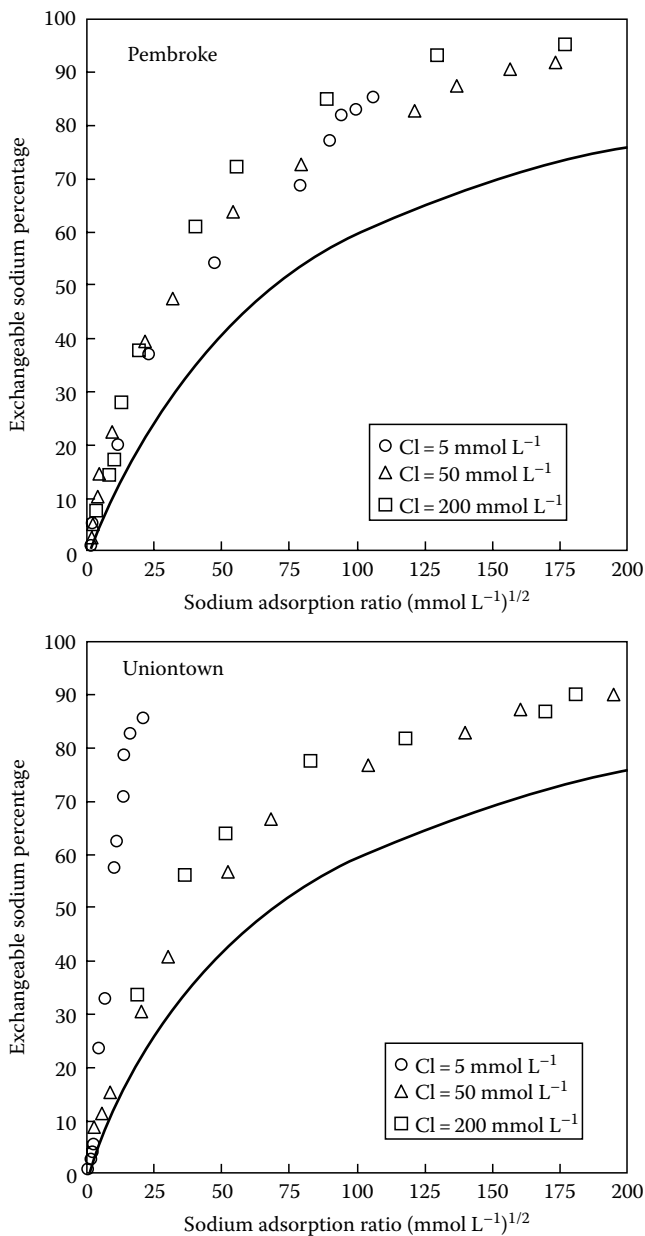


**FIGURE 4.3** Relationship between ESP and SAR at a chloride (Cl) concentration of 5 mmol L<sup>-1</sup> of Pembroke and Uniontown soils at three pH values. The solid line without data represents most salt-affected soils in the western United States. (Redrawn from Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, A26(7), 1147, 1991a. With permission.)

exchange (soil sodication) followed by an increase in CEC with increases in pH. In the study by Sreenivas and Reddy (2008), variations in soluble Ca<sup>2+</sup> ion concentrations were negatively correlated to pH and positively correlated to salinity. The two parameters, pH and salinity, were negatively correlated.

The ESP versus SAR plots of the Pembroke soils are presented in Figures 4.3 and 4.4. These two figures demonstrate that the ESP–SAR relationship of the Pembroke soil is independent of pH and ionic strength. The data also imply that the  $K_v$  for this soil should be independent of pH and ionic strength (Evangelou and Phillips, 1987). These data also point out that at low ESP values (ESP < 20) the soil exhibits a high affinity for Na<sup>+</sup>, perhaps because of steric processes. At ESP > 20, no ion preference (Sposito, 1984) is implied. Furthermore, it appears that the Pembroke soil behaves as an ideal exchanger between ESP of about 20 and 100. These observations are consistent with the information presented by van Bladel et al. (1987) and Levy and Hillel (1968).

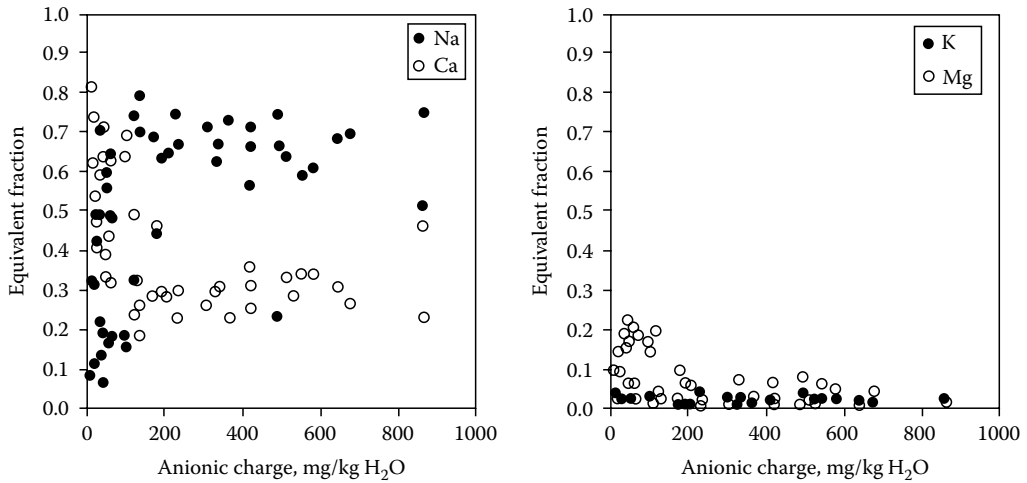
The apparent lack of influence of pH and ionic strength on the selectivity of such soils for either Na<sup>+</sup> or Ca<sup>2+</sup> could be related to a number of processes that take place on a clay surface as pH and/or ionic strength increases. For example, Pratt et al. (1962) have demonstrated on a number of soils that



**FIGURE 4.4** Relationship between ESP and SAR at three chloride (Cl) concentrations of the Pembroke and Uniontown soils at pH 4.3. The solid line without data represents most salt-affected soils in the western United States. (Redrawn from Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, A26(7), 1147, 1991a. With permission.)

as pH decreases the exchange selectivity coefficient of Na<sup>+</sup>–Ca<sup>2+</sup> exchange increases. This increase signifies increase in affinity of the Na<sup>+</sup> by the clay surface through decreasing surface charge density. The data by Pratt et al. (1962) tend to support this conclusion. Additionally, Shainberg et al. (1980) have shown that for Na<sup>+</sup>–Ca<sup>2+</sup> exchange, as ionic strength increases the affinity for Na<sup>+</sup> by the illite surface also increases. The latter observation, however, depends, on whether one deals with an external surface or internal surface. For example, an increase in ionic strength on an external surface (low electric potential surface) could increase the affinity for the Ca<sup>2+</sup>. However, an increase





**FIGURE 4.5** Variation in the equivalent fraction of Na, Ca, K, and Mg adsorbed on the sediment as a function of total dissolved anionic charge. The total range of salinities represented here is from approximately 580–53,000 mg/L. Note the shift from Ca as the predominant adsorbed cation to Na as total dissolved anionic charge increases. (Redrawn from Hanor, J.S., *Appl. Geochem.*, 22(10), 2115, 2007.)

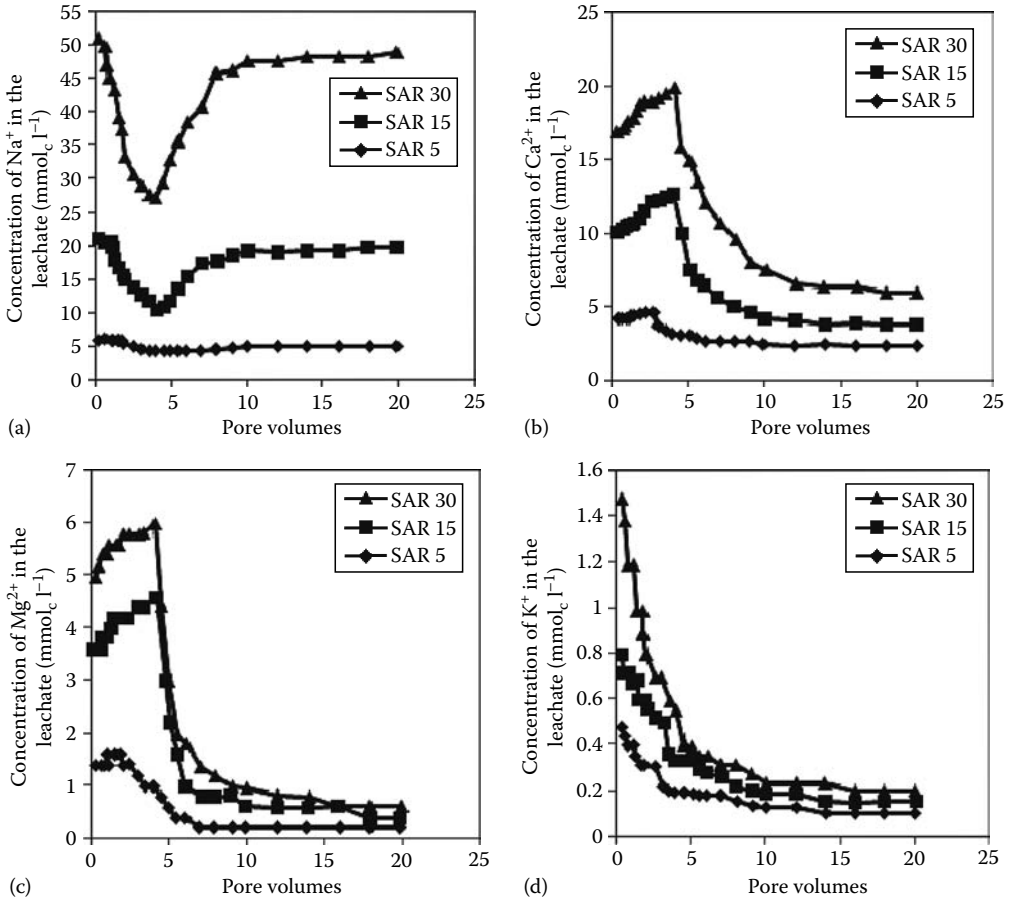
in ionic strength on an internal surface (high electric potential surface) could increase the affinity for the Na<sup>+</sup>. Considering that mixed mineralogy soils are made up of external and internal surfaces, a canceling effect on the magnitude of surface selectivity due to an increase in ionic strength could be obtained.

The ESP versus SAR plots for the Uniontown soil are also shown in Figures 4.3 and 4.4. That pH and ionic strength have a strong influence on  $K_v$  is strongly supported by these figures. As pH and ESP increased, Na<sup>+</sup> is preferred by the solid phase. Stumm and Bilinski (1973) showed that deprotonating clay edge surfaces have greater affinity for a monovalent cation than a divalent cation, because the former (monovalent cation) requires much less free energy to desolvate and thus come closer to the adsorbing surface. On the other hand, according to the data shown in Figure 4.4, as ionic strength increases, the preference of the solid phase for Na<sup>+</sup> decreases. This also implies that under high ionic strength the divalent cations are most likely to carry out the soil deprotonation process. Finally, the data in Figures 4.3 and 4.4 clearly demonstrate that the soils in the two humid regions exhibit a much less affinity for Na<sup>+</sup> than the average salt-affected soil in the western United States. Note the difference in the Na<sup>+</sup> adsorption isotherms exhibited by the humid region soil and the western U.S. soils. This implies that physical behavior and reclamation practices for these two groups of soils are expected to be different.

Hanor (2007) studied the effects of salinity (ionic strength) on the composition and partitioning of adsorbed cations onto clays from brine-contaminated soil samples from the siliciclastic sediments in Southern Louisiana. At low salinities (<1200 mg L<sup>-1</sup>), Ca and Mg dominated as adsorbed cations. At moderate to high salinities (up to 53,000 mg L<sup>-1</sup>), adsorbed sodium was dominant (Hanor, 2007). The relationship between the proportions of adsorbed cations salinity followed a nonlinear pattern and the transition from Ca- to Na-dominated adsorption occurred over a narrow range of salinities (Figure 4.5).

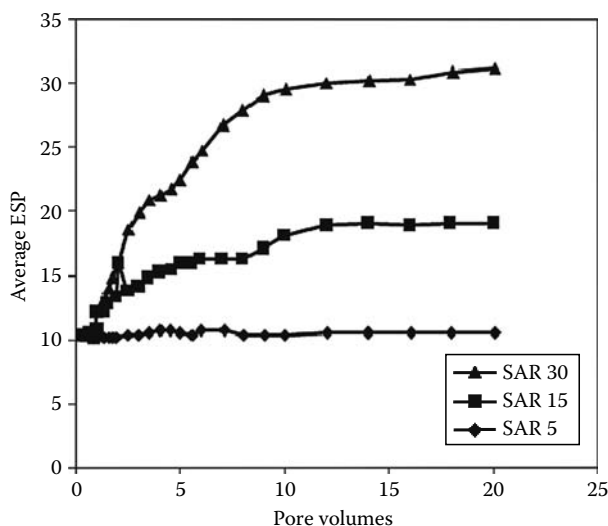
### 4.3.3 SODIUM CONCENTRATION EFFECTS

In sandy soils, the application of three types of irrigation waters with corresponding SAR values of 5, 15, and 30 resulted in the displacement of cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, and K<sup>+</sup>) and anions into solution and their replacement by Na<sup>+</sup>. Figure 4.6 shows the breakthrough curves for Na<sup>+</sup>, Ca<sup>2+</sup>,



**FIGURE 4.6** Breakthrough curves for (a) Na<sup>+</sup>, (b) Ca<sup>2+</sup>, (c) Mg<sup>2+</sup>, and (d) K<sup>+</sup> in soil leached with different SAR solutions. (Redrawn from Jalali, M. and Merrihpour, J., *Environ. Geol.*, 53, 1289, 2008.)

Mg<sup>2+</sup>, and K<sup>+</sup>. In Figure 4.6a, the concentration of effluent Na<sup>+</sup> decreased sharply to a minimum of about 4, 10.2, and 27 mmol<sub>c</sub> L<sup>-1</sup> for SAR 5, 15, and 30, respectively and reached input concentrations after about 15 pore volumes of leachate passed through the columns in all solutions. This decrease in effluent Na<sup>+</sup> concentration was accompanied by an increase in effluent Ca<sup>2+</sup> (Figure 4.6b) and Mg<sup>2+</sup> (Figure 4.6c) concentrations, probably resulting from the displacement of soil indigenous exchangeable Ca<sup>2+</sup> and Mg<sup>2+</sup> by leachate Na<sup>+</sup>. These peaks of displaced Ca<sup>2+</sup> and Mg<sup>2+</sup> were followed by sharp reduction in effluent concentrations, indicating slow release of both cations to soil solution and after about 20 pore volumes, reached approximately input values (Figure 4.6b and c). Leaching of Ca<sup>2+</sup>, Mg<sup>2+</sup>, and K<sup>+</sup> from soils subjected to irrigation with poor quality water presents a significant loss of valuable plant nutrient. In all treatments, effluent K<sup>+</sup> concentrations were drastically reduced and reached constant values after about five pore volumes of leachate (Figure 4.6d). After 20 pore volumes, the concentration of leached K<sup>+</sup> remained below 0.2 and 0.1 cmol<sub>c</sub> L<sup>-1</sup> for SAR 30 and 5, respectively (values above the water quality standards). An increase in K<sup>+</sup> leaching is therefore expected to increase K<sup>+</sup> concentrations in groundwater in agricultural areas (Jalali and Merrihpour, 2008). Also, increasing the level of SAR from 5 to 15 and 30 corresponded to an increase in the average ESP of the soil from 10.4 to 20.3, and 32.5, respectively (Figure 4.7), and resulted in dispersion of soil aggregates and reduction in soil permeability (Jalali and Merrihpour, 2008).



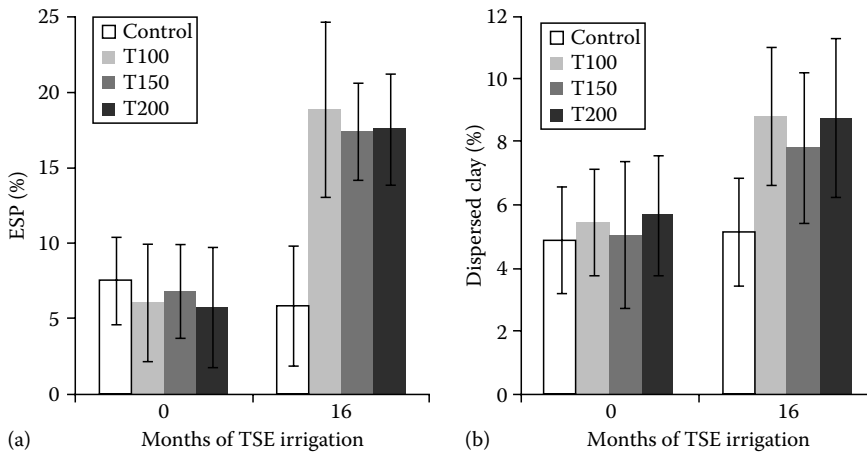
**FIGURE 4.7** Changes in average ESP as a function of pore volumes when soil was leached with different SAR solutions. (Redrawn from Jalali, M. and Merrikhpour, J., *Environ. Geol.*, 53, 1289, 2008.)

#### 4.4 SODIUM INFLUENCE ON SOIL DISPERSION AND SATURATED HYDRAULIC CONDUCTIVITY

When the amount of exchangeable  $\text{Na}^+$  relative to exchangeable  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  exceeds a critical threshold, there can be deleterious effects on physical properties of soil. Due to its large size and single charge, the adsorption of the hydrated  $\text{Na}^+$  ion into the diffuse double layer around the clay platelets cause them to separate (Curtin et al., 1994). This physical separation of clay platelets causes modification of soil pore distribution, results in high swelling pressure, and creates single clay platelets that persist in solution and/or are carried by water and deposited in soil pores (So and Aylmore, 1993; Mace and Amrhein, 2001). Clay dispersion in turn affects soil hydraulic conductivity. An increase in Na levels in the soil solution or on exchanging surfaces causes soil-saturated hydraulic conductivity to decrease (Russo and Bresler, 1977; Agassi et al., 1981; Kazman et al., 1983).

Soils disperse only when they are in equilibrium with an electrolyte solution concentration under the “flocculation value.” The flocculation value depends on SAR, solution ionic strength, clay mineralogy (El-Swaify, 1976), and pH (Keren et al., 1988). For example, flocculation values for Na/Ca–montmorillonite were 3, 4, and 7  $\text{mmol}_c \text{L}^{-1}$  and 6, 10, and 18  $\text{mmol}_c \text{L}^{-1}$  for Na/Ca–illite with ESP values of 5, 10, and 20, respectively (Shainberg and Letey, 1984). Under tropical conditions, irrigation with treated sewage effluents resulted in average increases of exchangeable sodium from 2.4 to 5.9  $\text{mmol}_c \text{kg}^{-1}$ , soluble Na from 1.4 to 4.7  $\text{mmol L}^{-1}$ , and SAR of soil solution from 3.6 to 12.6 ( $\text{mmol L}^{-1})^{1/2}$  in the soil profile (Leal et al., 2009). The increasing levels in these sodicity parameters resulted in significant increases in ESP and in clay (mostly kaolinite) dispersion rates (Figure 4.8). Based on these results, it was suggested that the negative effects of high SAR solutions on kaolinitic soils are more pronounced compared to the montmorillinitic soils of arid and semi-arid regions (Leal et al., 2009). Also, these soils are expected to have higher sensitivity to small increases in exchangeable Na and pH due to the presence of organic matter adsorbed to kaolinitic edges. This condition can result in the reversion of positive charges to negative charges and lead to the inhibition of the dominant edge-face flocculation (Leal et al., 2009 and references therein).

The classic theory of colloidal stability developed by Derjaguin and Landau (1941) and Verwey and Overbeek (1948) (DLVO theory) generally accounts for the influences of ion valence and concentration on suspended colloid interactions. According to the DLVO theory, the long-range

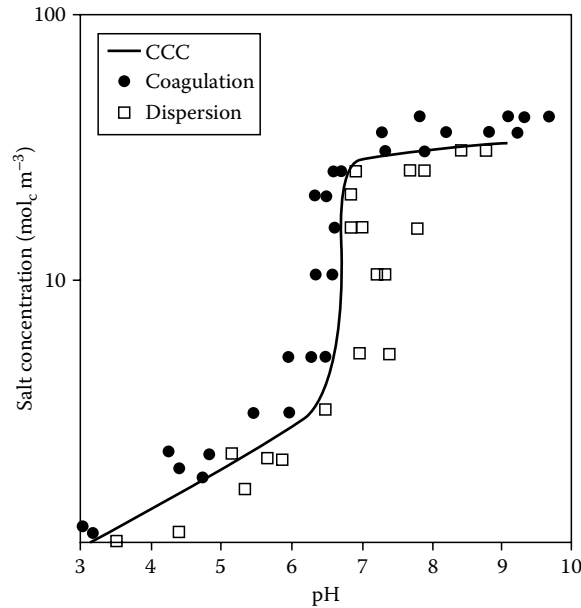


**FIGURE 4.8** Mean exchangeable sodium percentage (a) and mean percent clay dispersion (b) in the soil profile (0–100 cm) before and after TSE irrigation between the different irrigated treatments (T100, T150, and T200). Control, without TSE irrigation, and T100, T150, T200 corresponding to TSE irrigation supplying 100%, 150%, and 200% of the crop water demand, respectively. 0 month represents soil conditions prior to sugarcane planting and irrigation and 16 months correspond to soil conditions after 16 months of TSE irrigation. (Redrawn from Leal, R.M.P. et al., *Agric. Water Manage.*, 96(2), 307, 2009.)

repulsive potential resulting from diffuse double layers (DDLs) of like charged colloids retards the coagulation or flocculation rate of clay colloids.

Colloidal stability (maximum dispersion) depends on maximum  $\Phi$  ( $\Phi_{\max}$ ), which describes the maximum repulsive energy between two planar colloidal surfaces. Furthermore,  $\Phi_{\max}$  is controlled by surface electric potential ( $\Psi$ ) and ionic strength. The component,  $\Psi$ , is controlled by the pH of the colloidal suspension, assuming that the colloids exhibit pH-dependent charge (Uehara and Gillman, 1980). Generally, in clay colloids, on increasing pH,  $\Psi$  becomes more negative and thus  $\Phi_{\max}$  increases. Conversely, on decreasing pH,  $\Psi$  becomes less negative. When  $\Psi$  approaches zero,  $\Phi_{\max}$  approaches zero. This leads to colloid coagulation or flocculation (Emerson, 1964; Tama and El-Swaify, 1976; Singh and Uehara, 1986; Keren and Singer, 1988). Increasing  $I$  in a colloidal suspension decreases  $\Phi_{\max}$ , which enhances colloid flocculation rate (Emerson, 1964). Nasser and James (2008) demonstrated that at pH < 7 and regardless of electrolyte concentration, kaolinite-NaCl dispersions were highly flocculated with strong edge–edge interactions. Higher pH values and low electrolyte concentrations caused the flocculated structure of kaolinite to breakdown, whereas high electrolyte concentrations produced randomly structured flocs. Ahmad and Karube (2001) showed that the critical coagulation concentration (CCC); a threshold concentration between dispersion and coagulation that is comparable to the flocculation value and expressed in units  $\text{mol}_c \text{ kg}^{-1}$  of a montmorillonite clay decreased with decreasing pH (Figure 4.9). In NaCl, CCC decreased slightly as pH decreased from 10 to 7, sharply between pH 7 and 6, and then moderately as pH decreased from 6 to 3 (Figure 4.9). Similar CCC values were measured at pH below 6.5 in NaCl, KCl, and  $\text{NH}_4\text{Cl}$  solutions. At pH = 9, CCC followed the order of hydrated ion radius ( $\text{Na} > \text{K} > \text{NH}_4$ ). Also, the investigators (Ahmad and Karube, 2001) demonstrated that montmorillonite dispersed at low SAR value (below 13) when EC was less than a threshold value.

In addition to these components ( $\Psi$ ,  $I$ ) controlling colloidal flocculation or stability (Schofield and Samson, 1954; Evangelou, 1990; Shainberg and Letey, 1984), additional components in the case of clay colloids are also involved. These additional components include relative proportion of monovalent to divalent cations in the bulk solution (Shainberg and Letey, 1984), type of cations, shape of particles and initial particle concentration in suspension (Oster et al., 1980), type of clay minerals present, and relative proportion of clay minerals (McBride, 1981).



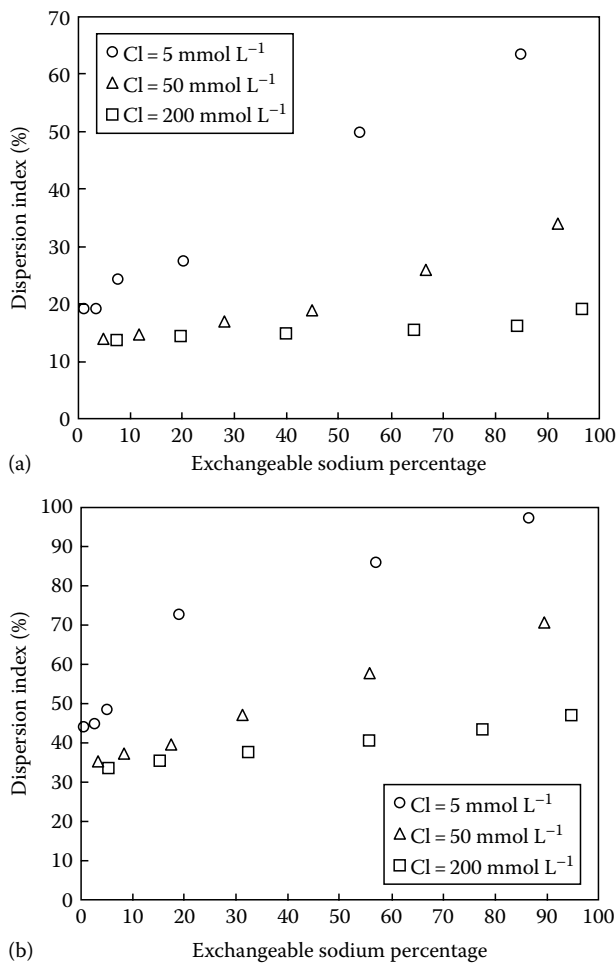
**FIGURE 4.9** Dispersion and coagulation of montmorillonite in NaCl solution at different pH. (Redrawn from Ahmad, M.M. and Karube, J., *Pak. J. Soil Sci.*, 20(4), 75, 2001.)

The above observations of the effect of clay mineral type and their relative proportion on dispersion and flocculation behavior suggest that certain interactions between the various colloids change their dispersive behavior or colloidal stability. Based on these observations, soils of mixed mineralogy and with various proportions of different clay minerals are expected to have unique dispersive properties.

Many processes and/or conditions in the soil environment are highly dependent on colloid dispersion or flocculation. Such processes and/or conditions include erosion, water suspension of solids, soil structure, and hydraulic conductivity, among many others. A number of studies involving sodic soils have been carried out in order to relate soil dispersive properties to saturated hydraulic conductivity. For example, Suarez et al. (1984) was able to link soil dispersion in suspensions measured spectrophotometrically to saturated hydraulic conductivity. Others researchers measured the percentage of clay in suspension (dispersion index) versus saturated hydraulic conductivity. The purpose in establishing clay dispersion–saturated hydraulic conductivity relationships is to develop rapid tests for predicting hydraulic conductivity of salt-affected soils and/or to evaluate mechanisms that are involved in regulating saturated hydraulic conductivity.

The data in Figure 4.10 show that the potential of soils to undergo dispersion is related to ESP. This is true only at low ionic strengths. When ionic strength was adjusted to 200 mmol L<sup>-1</sup>, there appears to be no effect of ESP on soil dispersion due to suppression of the double layer repulsive forces. These data are consistent with qualitative predictions of clay dispersion equations (Marsi and Evangelou, 1991b). The data in Figure 4.11 also show that, even at pH 4.3, both soils exhibit dispersion at low ionic strength. This observation suggests that at pH 4.3 both of these soils will likely exhibit a net negative charge.

It can be summarized from Figures 4.10 and 4.11 that the Uniontown soil was more sensitive to dispersion under decreasing electrolyte concentration and increasing ESP but less sensitive to pH changes than the Pembroke soil. The data also demonstrate that for any given electrolyte concentration, pH, and ESP, the dispersion index of the Uniontown soil was always greater than that of the Pembroke soil. This appeared to be in agreement with the thermodynamic exchange parameter of these soils. The magnitude of adsorbed ion activity coefficient  $f_{Na}$  for the Uniontown soil is greater



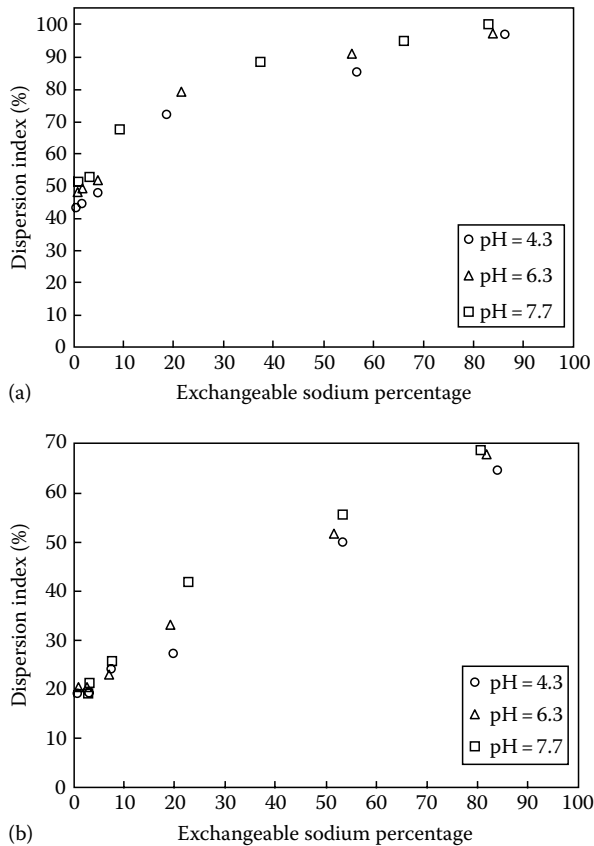
**FIGURE 4.10** Influence of ESP on the DI of the Pembroke (a) and Uniontown (b) soils near pH 4 and at three chloride (Cl) concentrations. (From Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, A26(7), 1177, 1991b. With permission.)

than 1 (data not shown). Considering that  $f_{Na} > 1$  could signify that  $Na^+$  “reside” in the diffuse layer, one expects the Uniontown soil to be highly dispersive. On the other hand, the magnitude of  $f_{Na}$  for the Pembroke soil is less than 1 (data not shown). Assuming this signifies that  $Na^+$  forms outer-sphere complexes with the clay surfaces, this soil would be expected to be less dispersive than the Uniontown.

The presence of exchangeable  $Na^+$  significantly decreases soil permeability (Quirk and Schofield, 1955; Reeve and Bower, 1960; Park and O’Connor, 1980). The mechanism(s) responsible for decreasing soil permeability in the presence of  $Na^+$  can be demonstrated by looking into the components controlling water or soil solution movement potential under saturating conditions.

Soil-saturated hydraulic conductivity is described by Lagerwerff et al. (1969) as

$$K = \frac{kg}{\eta} \tag{4.23}$$



**FIGURE 4.11** Influence of ESP on the DI of the Pembroke (a) and Uniontown (b) equilibrated with a solution of 5 mmol L<sup>-1</sup> chloride (Cl) at three pH values. (From Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, A26(7), 1177, 1991b. With permission.)

where

- $k$  is the permeability of the soil
- $g$  is the gravitational constant
- $\eta$  is the kinematic viscosity or the ratio of solution viscosity to fluid density

For soil systems contaminated with brackish solutions, kinematic viscosity is not significantly affected (Lagerwerff et al., 1969) and thus the components controlling water flow viscosity are the hydraulic gradient ( $H$ ) and soil permeability ( $k$ ). The latter component ( $k$ ) is influenced by clay dispersion and migration, and clay swelling. These processes may cause considerable alteration to soil matrix characteristics, such as porosity, pore-size distribution, tortuosity, and void shape (Cass and Sumner, 1982). A detailed description of the physicochemical mechanism influencing clay dispersion and/or clay swelling is given in Marsi and Evangelou (1991b).

The deterioration of soil physical properties influencing  $k$  is accelerated directly or indirectly by the presence of high Na<sup>+</sup> on the soil's exchange complex and the electrolyte composition and concentration of the soil solution (Quirk and Schofield, 1955; McNeal and Coleman, 1966, Rowell et al., 1969; Hamid and Mustafa, 1975; Yousaf et al., 1987). To improve soil physical properties of Na-affected soils, Ca<sup>2+</sup> is usually added to replace the Na<sup>+</sup> on the exchange sites. Calcium reduces clay swelling and enhances clay flocculation (Greacen, 1959).

Additional components influencing the effect of Na<sup>+</sup> on saturated hydraulic conductivity of soil include clay mineralogy, clay content, soil bulk density, Fe and Al oxide content, organic matter

content, salt concentration, and  $\text{Na}^+/\text{Ca}^{2+}$  ratio (Gardner et al., 1959; McNeal et al., 1968; Shainberg and Casserman, 1971; Frenkel et al., 1978; Cass and Sumner, 1982; Keren and Singer, 1988; Keren et al., 1988; Wada and Beppu, 1989). The hydraulic properties of soils dominated by 1:1 type clay mineralogy (i.e., kaolinite) and Fe and Al oxides are relatively insensitive to variations in soil solution composition and concentration in contrast to those dominated by 2:1 type clay minerals (i.e., montmorillonite). McNeal and Coleman (1966) stated that each soil has a unique saturated hydraulic conductivity response threshold because of its unique properties. Pal et al. (2006) suggest the use of saturated hydraulic conductivity of less than  $10\text{ mm h}^{-1}$  (weighted mean in 0–100 cm depth of soil) as a robust criterion instead of any ESP or SAR values.

Martin et al. (1964) studied the importance of pH on saturated hydraulic conductivity (SHC) and found that the same total quantity of  $\text{Na}^+$  on a soil will reduce SHC more effectively at a lower pH than at a higher pH. These investigators (Martin et al., 1964) concluded that the reduction in soil CEC as pH decreased was responsible for decreasing soil SHC, since the same amount of  $\text{Na}^+$  represents a greater ESP at a lower soil pH. Suarez et al. (1984) reported that for the same ESP or SAR value, the SHC decreased as pH increased. The pH effect on hydraulic conductivity is pronounced only when the soil contains a high quantity of variable-charge minerals and organic matter.

In contrast to the studies on the effect of the electrolyte concentration and composition on saturated hydraulic conductivity, fewer studies examined the influence of pH, solution composition, and salt concentration on SHC. It seems necessary to understand the influence of pH on soil hydraulic conductivity, because soils contaminated with oil well brine in humid regions are often associated with low pH; either pH drifts downward as extensive leaching is taking place or the pH rises when alkaline brines are discharged onto the soil.

Reductions in the relative saturated hydraulic conductivity (RSHC) as a function of pH and chloride concentration are summarized in Table 4.3. These data show “threshold” ESP or SAR values which are defined as 20% relative reduction in RSHC. It is clearly shown that the ESP–SAR critical threshold is highly dependent on pH and Cl concentrations. It varies from an SAR of approximately 0.30 to SAR of approximately 90. These values strongly indicate that the critical SAR threshold reported by the U.S. Salinity Laboratory Staff (1954), in the range of 10–15, applies to the  $50\text{ mmol L}^{-1}$  Cl concentration of the Uniontown soil only. The Pembroke soil (Table 4.3) at the  $50\text{ mmol L}^{-1}$  Cl concentration exhibits a more critical threshold.

The data presented in Table 4.3 show that the RSHC of the Uniontown soil is more sensitive to ionic strength and solution composition than that of the Pembroke soil. These sensitivity differences are probably a result of the differences in mineralogy between the two soils. The effect of clay mineralogy on the critical SAR was also reported by McNeal and Coleman (1966).

**TABLE 4.3**  
**SAR<sup>a</sup> and ESP Values Associated with 20% Reduction in RSHC for Pembroke and Uniontown Soils at Three pH Values**

| Cl (mmol L <sup>-1</sup> ) | Pembroke       |      |        |     |        |     | Uniontown      |      |        |     |        |      |
|----------------------------|----------------|------|--------|-----|--------|-----|----------------|------|--------|-----|--------|------|
|                            | pH 4.3         |      | pH 6.1 |     | pH 7.5 |     | pH 4.3         |      | pH 6.3 |     | pH 7.7 |      |
|                            | SAR            | ESP  | SAR    | ESP | SAR    | ESP | SAR            | ESP  | SAR    | ESP | SAR    | ESP  |
| 5                          | 2.6            | 5.5  | 1.6    | 1.1 | 0.4    | 0.6 | 2.4            | 2.8  | 1.1    | 0.8 | 0.3    | 0.5  |
| 50                         | 49.6           | 59.1 | 29.4   | 5.5 | 20.8   | 0.8 | 19.8           | 32.5 | 16.9   | 7.5 | 14.8   | 2.1  |
| 200                        | — <sup>b</sup> | —    | —      | —   | 90.4   | 0.5 | — <sup>b</sup> | —    | —      | —   | 83.2   | 68.5 |

Source: Marsi, M. and Evangelou, V.P., *Environ. Sci. Health*, A26(7), 1177, 1991b. With permission.

<sup>a</sup> SAR in  $(\text{mmol L}^{-1})^{-1/2}$ .

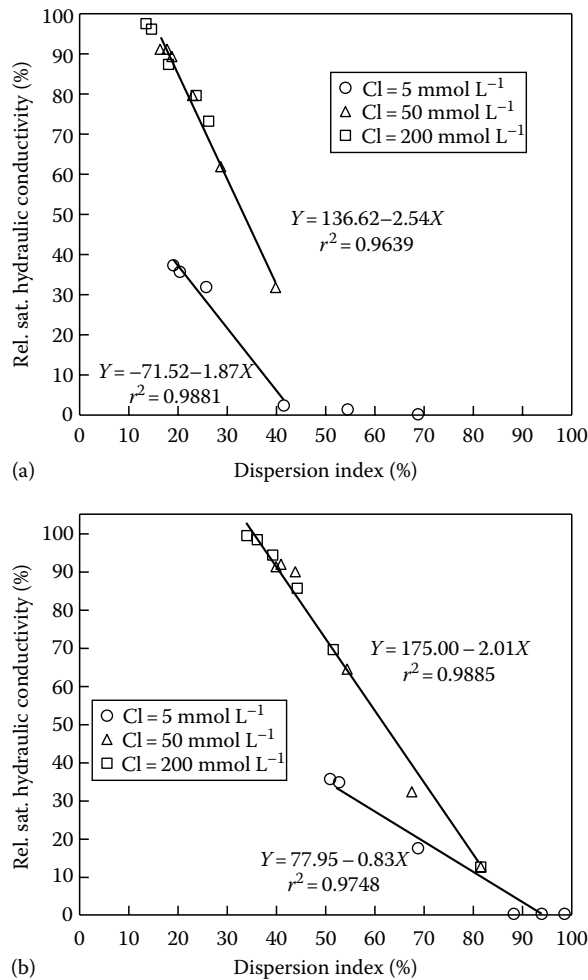
<sup>b</sup> Threshold values are not reported because the reduction on SHC is less than 20%.



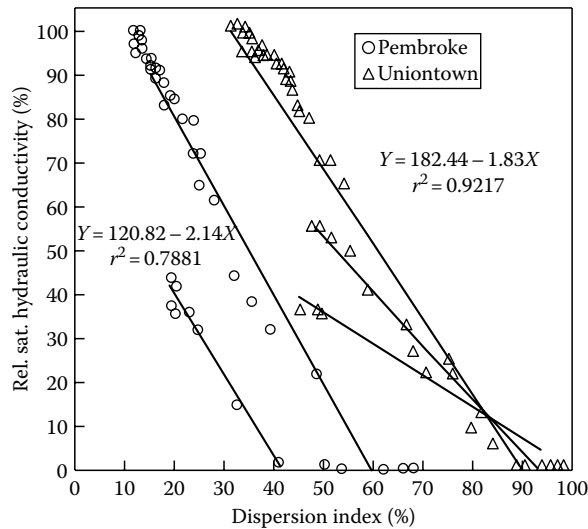
4.4.1 IMHOFF CONE-SATURATED HYDRAULIC CONDUCTIVITY

Values of RSHC correlated with Imhoff cone results, expressed as dispersion index (DI) to predict the RSHC for the soils, when salt affected, are shown in Figures 4.12 and 4.13.

Figure 4.12 demonstrates that for each of the two soils there was a unique RSHC–DI relationship at the 5 mmol L<sup>-1</sup> chloride concentration. More importantly, at this Cl concentration a lower DI was needed than with the higher Cl concentrations to suppress to a large degree the RSHC. This suggests that at the lower salt concentration, clay swelling is also implicated in reducing RSHC (Quirk and Schofield, 1955; McNeal and Coleman, 1966; McNeal et al, 1966; Rowell et al., 1969; Keren et al., 1988). In Figure 4.13, the slope of the RSHC–DI relationship was greater for the Pembroke soil than the Uniontown soil. This suggested that the RSHC of Uniontown soil was less affected by changes in DI than was the Pembroke soil. Moreover, these data also show that to attain similar relative suppression in RSHC, a greater DI was needed for the Uniontown soil than the Pembroke soil. This is probably due to soil texture. The Pembroke soil contained 59% clay, whereas the Uniontown contained only 28%. Hamid and Mustafa (1975) reported that RSHC–DI relationships are highly affected by soil texture as well as pore size distribution. It is also implied in



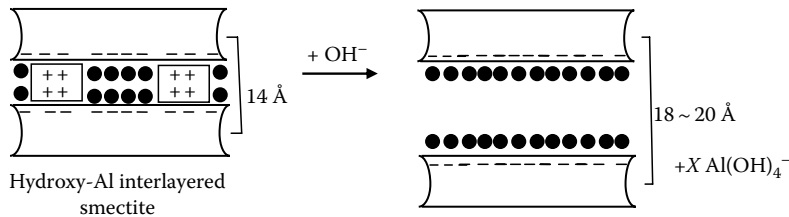
**FIGURE 4.12** Relationship between RSHC and DI of the Pembroke soil at pH 7.5 (a) and Uniontown soil at pH 7.7 (b) with three chloride (Cl) concentrations. (From Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, A26(7), 1195, 1991c. With permission.)



**FIGURE 4.13** Relationship between RSHC and DI of the Pembroke and Uniontown soils equilibrated with three chloride (Cl) concentrations at three pH values (DI = amount of dispersed clay divided by the amount of clay in 1 g soil). (From Marsi, M. and Evangelou, V.P., *J. Environ. Sci. Health*, 1991c. With permission.)

Figure 4.13 that as pH increases, a small DI imposes a large suppression in the RSHC. The increase in pH could be implicated in increasing swelling potential. This is likely because of the removal of Al-OH polymers from the interlayer (Figure 4.14). The presence of Al-OH polymers at the lower pH values may limit interlayer swelling (Barnhisel, 1977). Clays that have the basic 2:1 mineral structure may exhibit limited expansion because of the presence of hydroxy-Al islands which block their interlayer spaces (Figure 4.14). It is well known that these Al interlayer components are completely removed at pH values 9.0–10, through dissolution mechanisms (Keren, 1980). This interlayer removal is expected to increase the dispersion potential of the mineral by allowing free expansion. Similar phenomena of hydroxy-Al interlayer removal have been demonstrated to be the cause for failed septic systems (Zelazny et al., 1980), under a far less dramatic chemical regime than that often encountered in salt brine-contaminated systems. In addition to increased swelling, dispersion can also be enhanced in such systems as a result of the increased mineral surface charge following removal of Al-hydroxy from the interlayer. When removed from interlayer positions, these positively charged hydroxyl-Al components would increase the effective surface charge available for Na adsorption, thus increasing the probability of soil structural destabilization.

In conclusion the DI could predict RSHC. The relationship between RSHC and DI is not universal; however, it is unique to a particular soil under a given set of leaching conditions. The properties that appear to influence the RSHC–DI relationship of soils in humid regions are soil mineralogy, soil texture, soil pH, ionic strength, and solution composition. Information on humid region soils clearly demonstrates the following points: (1) the RSHC is related to the clay DI,



**FIGURE 4.14** Al-OH polymer removal from the interlayer space of 2:1 clay.

(2) the relationship between RSHC and DI is dependent upon ionic strength and pH; and (3) soils exhibit different RSHC–DI relationships. Furthermore, soils of the humid regions appear to behave differently with respect to  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange and physical stability in relationship to soils of arid regions.

4.5 ASSESSMENT OF SALINITY AND SODICITY

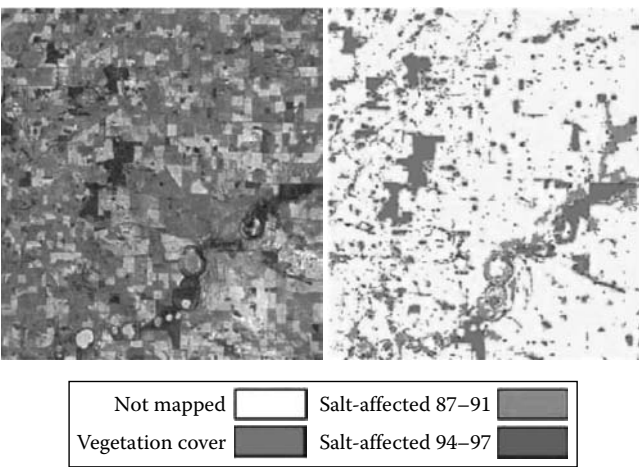
Different methods have been developed to identify, map, and monitor saline and sodic soils: (a) remote sensing, (b) solute transport modeling, and (c) geophysics. Other techniques such as spectroscopy (namely XRD, thin section microscopy, and SEM-EDS) have also been applied to recognize the presence and abundance of salt in soils (Farifteh et al., 2008; Wongpokhom et al., 2008).

4.5.1 REMOTE SENSING

Remote sensing data and technology have been widely used to map severely salt-affected areas (Goetz and Herring, 1989; Mougenot et al., 1993; Ben-Dor, 2002; Dehaan and Taylor, 2003). Compared to other methods of soil salinity assessment, remote sensing techniques save time, labor, and effort (Robbins and Wiegand, 1990). Remote sensors are of three types: (a) ground-based, (b) air-borne, and (c) space-borne. Air-borne and/or space-borne remotely sensed data are generally complemented with field measurement to determine the relations between spectral signature and soil surface properties (Figure 4.15).

An example of ground-based sensors is the spectroradiometers such as the GER3700 and FieldSpec FR (ASD). These instruments measure the reflectance spectra of salt-affected soils in narrow and continuous spectral bands, which are then analyzed using regression (monovariate, multivariate, and/or partial least squares) analyses and compared to pre-established minerals and salt crusts to identify and quantify salinity in salt-affected areas (Drake, 1995; Howari et al., 2002; Farifteh et al., 2004).

Airborne sensors such as AVIRIS provide high spectral and spatial resolution imageries that have been successfully used, in combination with field measurements, to map salt-affected areas with different degrees of severity (Chaturvedi et al., 1983; Taylor et al., 1996; Metternicht, 1998; Taylor and Dehaan, 2000; Ben-Dor, 2002; Dehaan and Taylor, 2002; Metternicht and Zinck, 2003). To identify saline soils, the collected radiance values are converted to reflectance values of the land surface (Clark et al., 2002). Space-born sensors provide global coverage of the earth’s surface



**FIGURE 4.15** Image classification of salt-affected soils using thematic mapper (TM) images (CSIRO, Canberra, Australian Capital Territory, Australia). (From Farifteh, J. et al., *Geoderma*, 145(3/4), 196, 2006.)

conditions at different spatial and temporal resolutions. The application of broadband remote sensing (e.g., Landsat, SPOT, Aster) in salinity studies has been limited due to spatial and spectral resolution problems that mask detailed spectral signatures (Cloutis, 1996). Spectral data are often integrated with GIS (Geographic Information Systems) and spatial statistics to map and model the spatial distribution of salinity (Chong et al., 2001; Khan et al., 2001).

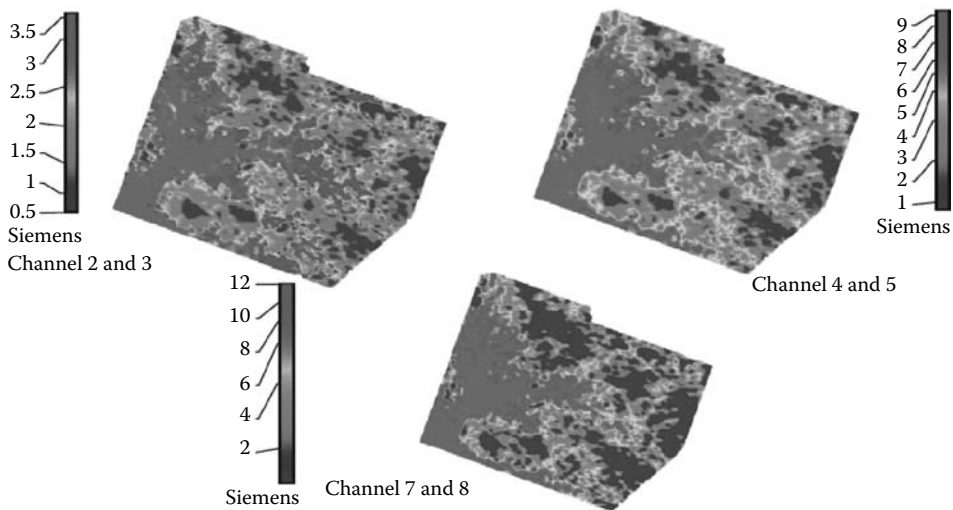
Metternicht and Zinck (2003) have critically reviewed the potentials and limitations of the application of remote sensing technology to the assessment of soil salinity. Remote sensors have been successfully used to detect very saline soils (Eldiery et al., 2005; Farifteh et al., 2006). The potential for remote sensing technology to recognize different degrees of soil salinity and/or sodicity is limited despite recent attempts by Bannari et al. (2008) to apply advance land imaging (EO-1 ALI) sensor spectral bands to discriminate between slightly and moderately saline soils. Remote sensors are unable to detect salt distribution in the near-surface or subsurface soils. Sensors are only able to scan the soil surface. Because the entire soil profile is involved in the salinization process and management, other solute transport modeling and geophysical techniques should also be used.

#### 4.5.2 SOLUTE TRANSPORT MODELING

Modeling solute transport provides spatial and temporal information on the movement of salt through the soil profile (Schoups and Hopmans, 2002). Solute transport modeling combines computer modeling, geostatistics, and field measurements (Jolly et al., 1998). Both mathematical and geopedologic models (e.g., Pishkar, 2003) have been used to study the mechanisms of solute transport in the vadose zone (Farifteh et al., 2006 and references therein). Other approaches have used transfer functions models to predict average values of solute concentration (as a function of depth and time) through highly variable field systems (Jury, 1982). El-Kadi et al. (1994) combined GIS with a numerical model to study solute transport in the geosphere.

#### 4.5.3 GEOPHYSICS

Geophysical methods are generally accepted as the most effective methods for the detection of soil salinity (Norman et al., 1988). Geophysical techniques are noninvasive and have been efficiently used to identify salinity in cropped land. A major advantage of geophysical technology is its ability



**FIGURE 4.16** Apparent conductance image of the Toolibin catchment. (From Farifteh, J. et al., *Geoderma*, 145(3/4), 196, 2006.)

to detect near-surface and subsurface salinity (Howlett et al., 2001). However, these technologies are expensive, time consuming, and require considerable human resources.

Geophysical instruments can be ground-, air-, and borehole-based instruments. Ground-based instruments are the most commonly used and are either electromagnetic (EM) conductivity meters or contact electrodes. Both instruments measure the apparent conductivity of soils ( $EC_a$ ) (Figure 4.16). This conductivity depends on salt concentration and type, clay content, soil moisture, and soil porosity. Applications of EM series instruments and their efficiencies in soil salinity assessments include the studies of Williams and Fiddler (1983), Rhoades et al. (1989), and Lesch et al. (1995). Paine et al. (2007) combined multifrequency EM induction spectral images with surface water chemical analysis to identify stream segments that receive saline flow in a small Texas coastal-plain basin.

#### 4.6 RECLAMATION OF SALT-AFFECTED SOILS

The removal of sodium from the soil profile of any given salt-affected soil is necessary, because Na is one of the most pronounced ions that influences plant growth and yield in salt-affected soils (Hoffman et al., 1983). Sodium is not only toxic to most plants because of its specific ion effect but it also influences certain soil properties. The major concern is the eventual deterioration of soil structure, resulting in decreased water infiltration and gas exchangeability (Bresler et al., 1982).

Management and reclamation of salt-affected soils are necessary to maintain, or increase, their productivity. At least three processes take place during reclamation of a sodic soil: (a) the  $Na^+$  on the exchange complex is replaced by  $Ca^{2+}$ ; (b) the soil-saturated hydraulic conductivity is improved following Ca application; and (c) sodium salts are removed from soil profile through leaching (James et al., 1982). Thus, reclamation of salt-affected soils often requires the removal of excess soluble salts as well as reduction of the soil ESP (see Oster and Frenkel, 1980 and references therein). The only proven method to reduce the soluble salt concentration in the root zone is through leaching. Reduction of the ESP is more difficult, because sodium ions adsorbed on exchange sites must first be replaced with divalent cations and then be leached from the root zone. According to Hoffman et al. (1983), the amount of leaching required is dependent on the salt content of irrigation water, salt tolerance of the crops, climatic conditions, and soil and water management practices.

Agromelioration is the approach of reclaiming saline and/or sodic soils in which the application of chemical amendments (such as hydrochloric acid, sulfuric acid, gypsum, etc.) is minimized and is compensated by agronomic practices (Zaka et al., 2003). In this approach, a starter dose of chemical amendment is added to bring the level of salinity/sodicity in the root zone of the salt-affect soil to a level within the tolerance limits of the crop. Further reclamation is obtained through leaching and cultural practices such as the application of organic matter. Theoretically, the addition of organic matter to saline soils of low CEC increases CEC and promotes the aggregation of soil particles. As a result, leaching is improved (Murtaza et al., 2001). Hussain et al. (2001) found that combining three amendments (gypsum, sulfuric acid, and farmyard manure) significantly decreases SAR of sodic soils and improves soil conditions (decreases bulk density, increases porosity, water permeability, and hydraulic conductivity). In another study by Kaur et al. (2008), the application of gypsum and organic matter (farmyard manure and green manure) decreased pH, EC, SAR, bulk density, and ESP and increased the water infiltration rate in a sandy loamy soil that has been subjected to 14 years of irrigation with sodic waters. Nevertheless, more research is needed to quantify the amount of organic matter required, in conjunction with inorganic amendments, to economically reclaim sodic soils. A review of the major lessons and observations acquired in the remediation of oil and brine spills in Oklahoma prairie are addressed by Sublette et al. (2007).

The most common method for determining soil salinity and/or sodicity in the laboratory consists of taking soil extracts and measuring parameters such as soluble and exchangeable ions, pH,  $EC_e$ , and SAR (Muhammad, 1996). From a practical standpoint, this method is time consuming and requires special equipment. To overcome this difficulty, researchers have used various soil to water ratios for obtaining extracts (Magistad et al., 1945; Chang, 1953). The variation in the soil to water ratio used

by investigators have led to erroneous and misleading results. Afzal and Yasine (2002), demonstrated that an increase in the moisture content of a soil sample (corresponding soil:water ratios of 1:1, 1:2, 1:5, and 1:10) was accompanied by an increase in the pH of the extract and the concentration of soluble ions ( $\text{Na}$ ,  $\text{Ca} + \text{Mg}$ ,  $\text{CO}_3 + \text{HCO}_3$ ,  $\text{SO}_4$ ) and a decrease in  $\text{ExNa} + \text{K}$ ,  $\text{ExCa} + \text{Mg}$ , and ESP. The authors (Afzal and Yasine, 2002) suggest the use of the saturated paste method for an accurate determination of pH, soluble salts, and exchangeable cations and thus for classification and reclamation of salt-affected soils (Afzal and Yasine, 2002).

## 4.7 CONCLUSIONS

The DI could predict RSHC. The relationship between RSHC and DI is not universal; however, it is unique to a particular soil under a given set of leaching conditions. The properties that appear to influence the RSHC–DI relationship of soils in humid regions are soil mineralogy, soil texture, soil pH, ionic strength, and solution composition. Information on humid region soils clearly demonstrates the following points: (a) The RSHC is related to clay DI, (b) the relationship between RSHC and DI is dependent upon ionic strength and pH, and (c) soils exhibit different RSCH–DI relationships. Furthermore, soils of the humid regions appear to behave differently with respect to  $\text{Na}^+ - \text{Ca}^{2+}$  exchange and physical stability in relationship to soils of arid regions.

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## *Part II*

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### *Plant/Crop Tolerance and Stressful Conditions*

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# 5 Oxidative Stress and Antioxidative Defense Systems in Plants Growing under Abiotic Stresses

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## 5.1 INTRODUCTION

The production of reactive oxygen species (ROS) is an unavoidable consequence of aerobic metabolism. ROS are highly reactive products that are generated due to the stepwise reduction of molecular oxygen ( $O_2$ ) by high-energy exposure or as a result of electron-transfer chemical reactions. ROS include free radicals as well as the nonradical molecules of high reactivity, such as superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ) etc. Dual roles for ROS in plants have been suggested, as they serve as key regulators of growth, development, and defense pathways, as well as at excessive levels they cause oxidative damage to biomolecules leading to toxicity in the cell (Mittler et al., 2004). Stressful conditions of the environment such as drought, salt stress, chilling, heat shock, excess levels of heavy metals, anaerobiosis, UV-B radiation, gaseous pollutants lead to the enhanced generation of ROS in plants due to the disruption of cellular homeostasis (Yan et al., 1996; Pellinen et al., 1999; Shah et al., 2001; Mittler, 2002; Pasqualini et al., 2002; Sharma and Dubey, 2005; Suzuki and Mittler, 2006; Esfandiari et al., 2008; Hu et al., 2008; Han et al., 2009; Kumutha et al., 2009; Maheshwari and Dubey, 2009; Sairam et al., 2009; Tanou et al., 2009). The enhanced production of ROS during abiotic stresses can pose a threat to cells by causing the peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately cell death (Shah et al., 2001; Mittler, 2002; Pasqualini et al., 2003; Verma and Dubey, 2003; Meriga et al., 2004; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey, 2009). ROS are viewed as the cellular indicators of stress as they serve as secondary messengers in the stress response signal transduction pathway. ROS also play an important role in lignification and other cross-linking processes in the cell wall (Bradley et al., 1992; Ogawa et al., 1997; Desikin et al., 2001; Mittler, 2002). Because of the multifunctional roles of ROS, it is necessary for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them completely. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system comprising of the nonenzymic as well as enzymic antioxidants (Noctor and Foyer, 1998). Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics serve as potent nonenzymic antioxidants within the cell. The enzymic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate–glutathione (AsA–GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer, 1998). However, the equilibrium between production and scavenging of ROS is perturbed under stressful conditions; therefore the modulation of antioxidant levels constitutes an important adaptive response to withstand adverse environmental conditions (Foyer et al., 1994; Noctor et al., 2002; Pitzschke and Hirt, 2006). Various workers have reported increased activities of many enzymes of the antioxidant defense system in plants to combat oxidative stress induced by drought (Rajamickam et al., 2005; Sharma and Dubey, 2005), soil salinity (Lin and Kao, 2000; Tsai et al., 2005; Tanou et al., 2009), high temperature (Rijstenbil et al., 1994; Almeselmani et al., 2006; Yin et al., 2008), chilling (Fryer et al., 1998; Bafeel and Ibrahim, 2008; Radyuk et al., 2009), metal toxicity (Shah et al., 2001; Verma and Dubey, 2003; Sharma and Dubey, 2007; Maheshwari and Dubey, 2009), anaerobiosis (Lin et al., 2008; Kumutha et al., 2009), gaseous pollutants (Ranieri et al., 1997; Scabbba et al., 2003), and UV-B radiation (Santos et al., 1999; Agarwal and Shaheen, 2007). The maintenance of a high antioxidant capacity to scavenge the toxic ROS has been linked to increased tolerance of plants to these abiotic stresses (Agarwal and Shaheen, 2007; Zaefyzadeh et al., 2009).

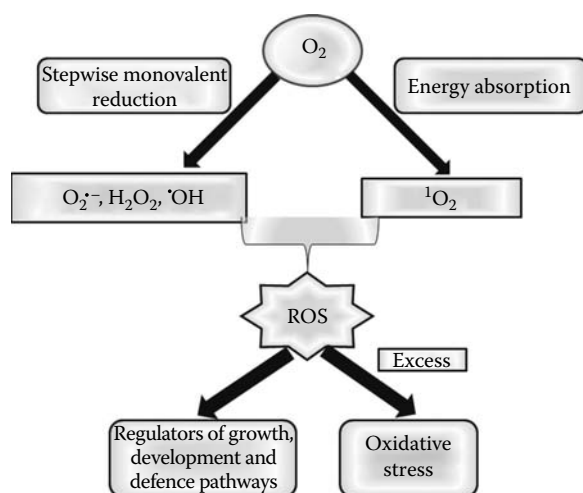
Potential to engineer plants that overexpress genes for antioxidants provides an opportunity to develop plants with enhanced tolerance to abiotic stress. With the advancements in molecular biology and the availability of advanced genetic tools, considerable progress has been made in improving stress-induced oxidative stress tolerance in crop plants by developing transgenic lines with altered levels of antioxidants (Allen et al., 1997; Lee et al., 2007; Ashraf et al., 2008; Prashanth et al., 2008). However, as the ROS detoxification system is very complex, changing one component

of the antioxidative defense system may or may not change the capacity of the pathway as a whole (Tseng et al., 2007; Lee et al., 2009). Further, the overexpression of the combinations of antioxidant enzymes in transgenic plants has been shown to have synergistic effects on stress tolerance. Therefore, increased emphasis is being given to produce transgenic plants overexpressing genes associated with more than one antioxidant in order to achieve tolerance to multiple environmental stresses.

The present review focuses on the abiotic stress-induced ROS production, oxidative damage caused by ROS, and the role of antioxidative defense systems in plants growing under a wide range of abiotic stresses. The progress made during the last few decades in improving abiotic stress tolerance in crop plants using genes responsible for the synthesis of antioxidants involving biotechnological approaches has also been discussed.

## 5.2 REACTIVE OXYGEN SPECIES AND INDUCTION OF OXIDATIVE STRESS

Like all aerobes, plants use  $O_2$  as terminal electron acceptor. At ambient temperature, one-step full reduction of  $O_2$  to  $H_2O$  could proceed very slowly due to the requirement of high activation energy. When oxygen is exposed to high-energy or electron-transfer chemical reactions, it gets converted to various highly reactive chemical forms collectively known as ROS. Although, it has been shown that some of the ROS may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins, all ROS are extremely harmful to organisms at high concentrations. Figure 5.1 shows various ROS that are produced either due to stepwise reduction of  $O_2$  or as a result of energy absorption. In plants, ROS are continuously produced by the inevitable leakage of electrons on to molecular oxygen from the electron transport activities of chloroplast, mitochondria, and plasma membrane (Pinto et al., 2003), or as a byproduct of various metabolic pathways such as respiration and photosynthesis (Foyer and Harbinson, 1994; Apel and Hirt, 2004; Tsukamoto et al., 2005). These ROS include highly reactive species such as  $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$ ,  $^1O_2$  etc., which disrupt the homeostasis of the organism by oxidatively damaging membrane lipids, proteins, chlorophylls, and nucleic acids (Kanazawa et al., 2000; Cassells and Curry, 2001; Jiang and Zhang, 2001; Sharma and Dubey, 2005, 2007; Konieczny et al., 2008; Maheshwari and Dubey, 2009).



**FIGURE 5.1** Activation of  $O_2$  occurs by two different mechanisms. Stepwise monovalent reduction of  $O_2$  leads to formation of  $O_2^{\cdot-}$ ,  $H_2O_2$ , and  $\cdot OH$ , whereas energy transfer to  $O_2$  leads to the formation of  $^1O_2$ . At low concentrations, ROS may function as regulators of growth, development, and as important signaling molecules altering gene expression and modulating the activity of defense pathways. However, at high concentrations, ROS are extremely harmful and cause oxidative stress.

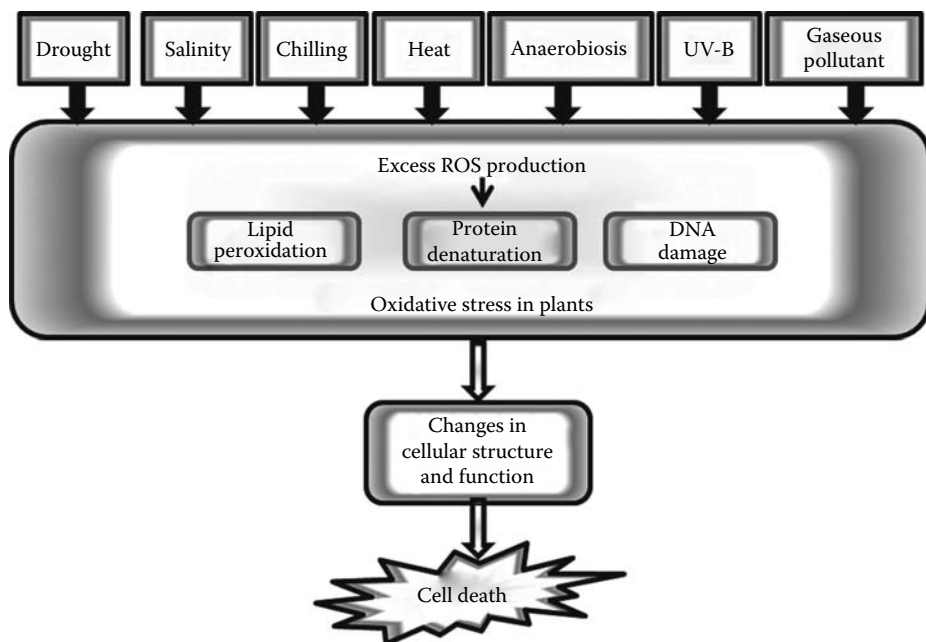
In its ground state, molecular  $O_2$  is relatively unreactive due to the presence of two unpaired electrons having parallel spin, which makes it paramagnetic (Apel and Hirt, 2004). Activation of  $O_2$  may occur by two different mechanisms: monovalent reduction or absorption of sufficient energy to reverse the spin on one of the unpaired electrons. The stepwise monovalent reduction of  $O_2$  leads to the formation of  $O_2^{\cdot-}$ ,  $H_2O_2$ ,  $\cdot OH$ , etc., whereas energy transfer to  $O_2$  leads to the formation of  $^1O_2$  (Figure 5.1). Singlet oxygen is a highly destructive ROS, which reacts with most of the biological molecules at near diffusion-controlled rates (Foyer and Harbinson, 1994). It is much more reactive toward organic molecules and can last for 4  $\mu s$  in water and 100  $\mu s$  in a nonpolar environment (Foyer and Harbinson, 1994).  $O_2^{\cdot-}$  is produced if one electron is added to ground state oxygen. Plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases use cytosolic NADPH to produce  $O_2^{\cdot-}$  under abiotic stress conditions.  $O_2^{\cdot-}$  is a moderately reactive, short-lived ROS with a half-life of approx. 2–4  $\mu s$ .  $O_2^{\cdot-}$  is a nucleophilic reactant with both oxidizing and reducing properties (Halliwell, 1977). With regard to the molecules of biological importance,  $O_2^{\cdot-}$  can oxidize sulfur compounds, O-diphenols, AsA (Mishra, 1974; Miller and MacDowell, 1975), cytochrome C (McCord et al., 1977), metal ions, and metal complexes (McCord and Day, 1978).  $H_2O_2$  is produced in the SOD-catalyzed disproportionation of  $O_2^{\cdot-}$ , or from the reduction of  $O_2^{\cdot-}$  by AsA, manganese ions, or ferredoxin (Hideg, 1997). The  $H_2O_2$  is one of the major and the most stable ROS that regulates basic acclimatization, defense, and developmental processes in plants (Slesak et al., 2007). It has been shown to act as a signal transduction molecule in several developmental processes. Nevertheless, at high concentrations, it causes oxidative stress marked by increased lipid peroxidation and the alteration of membrane permeability (Imlay, 2003). The main source of  $\cdot OH$  in biological systems is the decomposition of  $H_2O_2$  in the Haber–Weiss reaction. This reaction is enhanced by the presence of a transition metal, such as  $Fe^{2+}$  (Fenton reaction) (Hideg, 1997).  $\cdot OH$  is the most reactive among all ROS.  $\cdot OH$  can potentially react with all biological molecules, can initiate self-perpetuating lipid peroxidation, and, because cells have no enzymatic mechanism to eliminate this highly reactive ROS, its excess production would eventually lead to cell death (Pinto et al., 2003).  $\cdot OH$  interacts with all biological molecules and causes subsequent cellular damages such as lipid peroxidation, protein damage, and membrane destruction (Foyer et al., 1997). The well-known reactivity of  $H_2O_2$  is not due to its reactivity per se, but due to the formation of highly reactive  $\cdot OH$ , which is strong oxidizing agent, and is formed in the presence of metal reductants (Boo and Jung, 1999).

The production of ROS under normal growth conditions in cells is low (240  $\mu M s^{-1}$   $O_2^{\cdot-}$  and a steady state level of 0.5  $\mu M$   $H_2O_2$  in chloroplasts) (Polle, 2001). However, the various stressful conditions of the environment that disrupt the cellular homeostasis enhance the production of ROS (upto 720  $\mu M s^{-1}$   $O_2^{\cdot-}$  and a steady state level of 5–15  $\mu M$   $H_2O_2$ ) (Polle, 2001). When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of “oxidative stress.” Oxidative stress is defined as a shift of the balance between prooxidative and antioxidative reactions in favor of the former (Grzegorz, 1997). Figure 5.2 shows the enhanced production of ROS in plants growing under various abiotic stresses such as drought, salinity, extremes of temperature, excessive levels of metals, anaerobiosis, gaseous pollutants, and UV-B radiation. The enhanced level of these ROS causes oxidative damage to biomolecules such as membrane lipids, proteins, enzymes, nucleic acids, chloroplast pigments, etc. (Dat et al., 2000; Shah et al., 2001; Mittler, 2002; Pasqualini et al., 2003; Verma and Dubey, 2003; Meriga et al., 2004; Posmyk et al., 2005; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey, 2009).

### 5.2.1 LIPID PEROXIDATION

Lipid peroxidation is a normal metabolic process associated with the developmental processes of plants, including the juvenile stage of growth, the production of odor volatiles, senescence, and the formation of compounds like jasmonic acid under normal aerobic conditions (Anderson, 1995). Both free radicals as well as enzymes can lead to the initiation of lipid peroxidation in





**FIGURE 5.2** Various abiotic stresses like drought, salinity, extreme temperatures, heavy metals, anaerobiosis, gaseous pollutants, and UV-B radiation cause enhanced production of ROS in plants. These ROS cause oxidative damage to lipids, proteins, and nucleic acids, which in turn leads to changes in cellular structure and function and ultimately cell death.

both cellular and organellar membranes. Increased peroxidation (degradation) of lipids has been reported in plants growing under various stressful conditions such as drought (Sharma and Dubey, 2005; Esfandiari et al., 2008), salinity (Tanou et al., 2009), chilling (O’Kane et al., 1996), heat (El-Shintinawy et al., 2004), metal toxicity (Shah et al., 2001; Verma and Dubey, 2003; Sharma and Dubey, 2007; Maheshwari and Dubey, 2009), anaerobiosis (Blokhina et al., 1999; Kumutha et al., 2009), UV-B exposure (Yannarelli et al., 2006), gaseous pollutants (Puckette et al., 2007), etc. Increase in lipid peroxidation under these stresses parallels with the increased production of ROS. Lipid peroxidation, in both cellular and organellar membranes, takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals (Montillet et al., 2005). The level of lipid peroxidation has been widely used as an indicator of free radical mediated damage to cell membranes under stressful conditions. Malondialdehyde (MDA) is one of the final products of the peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (Halliwell and Gutteridge, 1989). Yamauchi et al. (2008) showed that MDA generated in chloroplasts from peroxidized linolenic acid modifies proteins in heat-stressed plants. Using linolenic acid-deficient mutants (*fad3fad7fad8* triple mutant), it was concluded that linolenic acid could be a major source of protein modification by MDA in heat-stressed plants (Yamauchi et al., 2008).

The polyunsaturated fatty acids (PUFA) present in membrane phospholipids are particularly sensitive to attack by  $\cdot\text{OH}$  and other oxidants. When PUFA in biomembranes are peroxidized, a great diversity of aldehydes is formed, some of which are highly reactive. The peroxidation of PUFA by ROS attack can lead to chain breakage and thereby increase in membrane fluidity and permeability. Phospholipids are essential components of the membrane that surrounds the cell as well as other cellular structures, such as nucleus and mitochondria, and therefore damage to phospholipids can affect the viability of the cells (Woessmann et al., 1999). There are two common sites of oxygen free radical attack on the phospholipid molecules—the unsaturated (double) bond between two carbon atoms that can be easily obtained in chemical reaction and interaction with other substances and

the ester linkage between glycerol and the fatty acid. It has been suggested that decrease in cell membrane stability or increase in membrane permeability reflects the extent of lipid peroxidation caused by ROS (Sairam et al., 2002).

### 5.2.2 PROTEIN MODIFICATION

The attack of ROS on proteins results in the site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge, and increased susceptibility of proteins to proteolysis. ROS may cause the modification of proteins in a variety of ways, some are direct and others indirect. Direct modification involves modulation of a protein's activity through nitrosylation, carbonylation, disulfide bond formation, and glutathionylation. Proteins can be modified indirectly by conjugation with the breakdown products of fatty acid peroxidation (Yamauchi et al., 2008). As a consequence of excessive ROS production, tissues injured by oxidative stress generally contain the increased concentrations of carbonylated proteins (Dean et al., 1993). The enhanced modification of proteins has been reported in plants under various stresses (Rao et al., 1995; Sharma and Dubey, 2005, 2007; Maheshwari and Dubey, 2009; Tanou et al., 2009). The amino acids in a peptide differ in their susceptibility to attack by ROS and the various forms of ROS differ in their potential reactivity.  $\cdot\text{OH}$  and alkoxyl radicals are mainly involved in the oxidation of proteins. Sulfur-containing amino acids and thiol groups specifically are very susceptible sites for attack by ROS. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form a disulfide bridge. Several heavy metals including Cd, Pb, and Hg have been shown to cause the depletion of protein bound thiol groups (Stohs and Bagchi, 1995). Cytosolic proteins have a tendency to maintain their cysteine residues in reduced and native form, whereas many secreted proteins have evolved to be more stable when their cysteines are joined in disulfide bonds. Thus, changes in the reducing environment of the cytosol can have profound effects on protein folding and activity. It seems likely that unwanted disulfide bonds are generated in the normal resident proteins of the cytosol during oxidative stress (Cabiscol et al., 2000). Alternatively, oxygen can add to a methionine to form methionine sulfoxide derivative. A protein methionine sulfoxide reductase has been detected in pea chloroplasts (Ferguson and Burke, 1992). This enzyme reduces the methionyl sulfoxide back to methionyl residues in the presence of thioredoxin (Brot and Weissbach, 1982). Some forms of free radical attack on proteins are not reversible, for example, the oxidation of iron-sulfur centers by  $\text{O}_2^{\cdot-}$  destroys enzymatic function (Gardner and Fridovich, 1991). In these cases, the metal (Fe) binds to a divalent cation-binding site on the protein. The metal (Fe) then reacts in a Fenton reaction to form a  $\cdot\text{OH}$  that rapidly oxidizes an amino acid residue at or near the cation-binding site of the protein (Stadtman, 1986). Tyrosine is readily cross-linked to form bityrosine products (Davies, 1987; Malencik and Anderson, 2003). Histidine, lysine, proline, arginine, threonine, and serine form carbonyl groups on oxidation (Stadtman, 1986). With the finding that these amino acids are oxidized to carbonyl derivatives, several methods have been developed to detect the carbonyl content of proteins and used to measure protein damage (Cabiscol et al., 2000). The amino acids cysteine, methionine, and histidine are especially sensitive to attack and oxidation by  $\cdot\text{OH}$ . Accordingly, enzymes in which these amino acids are located at positions that are critical to enzyme activity will become inactivated by the interaction of ROS.

Oxidized proteins serve as better substrates for proteolytic digestion and proteolytic pathways could provide a valuable line of "secondary antioxidant defense" (Cabiscol et al., 2000). A strong correlation has been demonstrated between increased hydrophobicity on the protein surface and the recognition and proteolytic degradation of oxidatively modified proteins. Many intracellular proteins are degraded by the multicatalytic proteinase complexes, also called proteasomes, in a nonlysosomal pathway (Cabiscol et al., 2000). It has been suggested that protein oxidation could

predispose it to ubiquitination, which in turn would be a target for proteasomal degradation. It seems that after a certain degree of oxidative damage, further damage causes a decrease in proteolytic susceptibility. Several studies have revealed that heavily oxidized proteins, extensively cross-linked and aggregated, are not only poor substrates for degradation but also can inhibit proteases from degrading other oxidized proteins (Grune et al., 1997). The removal of damaged proteins is necessary to prevent their accumulation, which could compromise the correct metabolism of any cell exposed to oxidative stress.

Specific proteins appear to be particularly vulnerable to oxidative carbonylation in the matrix of plant mitochondria; these include several enzymes of the Krebs cycle, glycine decarboxylase, SOD, and heat shock proteins. Plant mitochondria contain a number of different proteases, but their role in removing oxidatively damaged proteins is, as yet, unclear (Moller and Kristensen, 2004). Among the techniques developed for the identification of oxidative stress-induced modifications of proteins, the so-called redox proteome, proteomics appears to be the best-suited approach. Oxidative stress leaves different footprints in the cell in the form of different oxidatively modified components and, using the redox proteome, it will be possible to decipher the potential roles played by ROS-induced modifications in stressed cells (Rinalducci et al., 2008).

### 5.2.3 DNA DAMAGE

DNA is cell's genetic material and any damage to the DNA can result in changes (i.e., mutation) in the encoded proteins, which may lead to malfunctions or the complete inactivation of the encoded proteins. Thus, it is essential for the viability of cell that the DNA remains intact. ROS are a major source of DNA damage (Imlay and Linn, 1986) and cause strand breaks, removal of nucleotides, and a variety of modifications in the organic bases of the nucleotides. Changes in the nucleotides of one strand can result in mismatches with the nucleotides in the other strand, yielding subsequent mutations. Although cells have developed repair mechanisms to correct naturally occurring changes in the DNA, additional or excessive changes caused by ROS or other agents can lead to permanent damage to the DNA with potentially detrimental effects for the cell (Chen et al., 2002). Mitochondrial and chloroplast DNA are more susceptible to oxidative damage than nuclear DNA due to the lack of protective protein, histones, and close locations to the ROS-producing systems in the former (Richter, 1992). Enhanced DNA degradation has been observed in plants exposed to various environmental stresses such as salinity (Liu et al., 2000), metal toxicity (Meriga et al., 2004), and gaseous pollutants (Pasqualini et al., 2003).

Both the sugar and base moieties are susceptible to oxidation by ROS. The degradation of bases produces numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine, and adenine ring-opened and saturated products (Tsuboi et al., 1998). 8-Hydroxyguanine is the most commonly observed product. The principle cause of single-strand breaks is the oxidation of the sugar moiety by the  $\cdot\text{OH}$ . Under physiological conditions, neither  $\text{H}_2\text{O}_2$  alone nor  $\text{O}_2^{\cdot-}$  can cause *in vitro* strand breakage. Therefore, it was concluded that the toxicity associated with these ROS *in vivo* is most likely the result of Fenton reaction. Imlay and Linn (1986) concluded from their studies on *E. coli* mutants that a Fenton-active metal is probably chelated to phosphodiester linkage in DNA. When this metal is reduced by NADP(H) or  $\text{O}_2^{\cdot-}$ , it will react with  $\text{H}_2\text{O}_2$  to form the  $\cdot\text{OH}$  (Imlay and Linn, 1986).  $\cdot\text{OH}$  then oxidizes an adjacent sugar or base causing the breakage of the DNA chain. When  $\cdot\text{OH}$  attacks either DNA or proteins associated with it, DNA protein cross-links are formed (Oleinick et al., 1986). DNA protein cross-links cannot be readily repaired and may be lethal if replication or transcription precedes repair.

$\cdot\text{OH}$  reacts with free carbohydrates, such as sugars, and polyols (Smirnoff and Cumbe, 1989). The oxidation of sugars with  $\cdot\text{OH}$  often releases formic acid as the main breakdown product (Isbell et al., 1973). Plant cell wall polysaccharides have been shown to be susceptible to oxidative scission mediated by  $\cdot\text{OH}$  *in vitro* under physiologically relevant conditions (Fry, 1998).

### 5.3 ANTIOXIDATIVE DEFENSE SYSTEM IN PLANTS

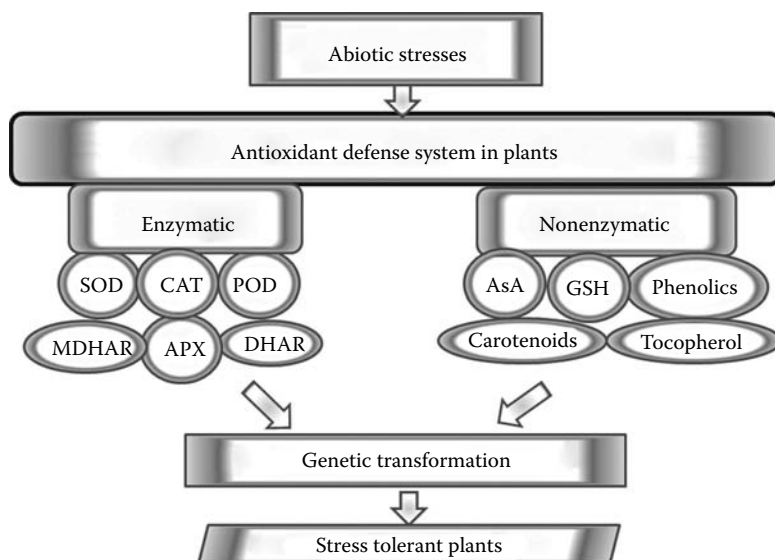
The balance between production and quenching of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels (Foyer et al., 1994; Noctor et al., 2002; Pitzschke and Hirt, 2006). The accumulation of ROS during abiotic stresses induces oxidative damage to lipids, proteins, and nucleic acids. Table 5.1 describes various plant species examined, in which different abiotic stresses cause overproduction of ROS leading to oxidative damage. In order to avoid the oxidative damage, higher plants possess a complex antioxidative defense system comprising of nonenzymatic and enzymatic components. A detailed account of these components has been presented in Figure 5.3. In plant cells, specific ROS-producing and scavenging systems are found in different organelles such as chloroplasts, mitochondria, and peroxisomes, and the ROS-scavenging pathways from different cellular compartments are coordinated (Pang and Wang, 2008).

#### 5.3.1 NONENZYMATIC DEFENSE SYSTEM

Nonenzymic components of the antioxidative defense system include the major cellular redox buffers AsA and GSH as well as tocopherol, carotenoids, and phenolic compounds. They interact with numerous cellular components and, in addition to crucial roles in defense and as enzyme cofactors, these antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (de Pinto and De Gara, 2004; Potters et al., 2004; Tokunaga et al., 2005). Mutants with decreased AsA, GSH, and tocopherol content have been shown to be hypersensitive to stress (Howden et al., 1995; Conklin et al., 1996; Huang et al., 2005; Semchuk et al., 2009).

**TABLE 5.1**  
**Abiotic Stresses Overproduce ROS and Cause Oxidative Damage to Different Plant Species**

| Abiotic Stress     | Plants                     | References                    |
|--------------------|----------------------------|-------------------------------|
| Drought            | <i>Oryza sativa</i>        | Sharma and Dubey (2005)       |
|                    | <i>Triticum aestivum</i>   | Esfandiari et al. (2008)      |
| Salinity           | <i>Sorghum bicolor</i>     | Hefny and Abdel-kader (2009)  |
|                    | <i>Fragaria ananassa</i>   | Tanou et al. (2009)           |
| Heat               | <i>Hordeum vulgare</i>     | El-Shintinawy et al. (2004)   |
|                    | <i>Lilium longiflorum</i>  | Yin et al. (2008)             |
| Chilling           | <i>Zea mays</i>            | Prasad et al. (1994)          |
|                    | <i>Cucumis sativus</i>     | Hu et al. (2008)              |
| Heavy metals       | <i>Oryza sativa</i>        | Shah et al. (2001)            |
|                    |                            | Verma and Dubey (2003)        |
|                    |                            | Sharma and Dubey (2007)       |
|                    |                            | Maheshwari and Dubey (2009)   |
|                    | <i>Nicotiana tabacum</i>   | Zrobek-Sokolnik et al. (2009) |
| Anaerobiosis       | <i>Hordeum vulgare</i>     | Kumutha et al. (2009)         |
|                    | <i>Zea mays</i>            | Jamei et al. (2009)           |
|                    |                            |                               |
| Gaseous pollutants | <i>Betula pendula</i>      | Pellinen et al. (1999)        |
|                    | <i>Medicago truncatula</i> | Puckette et al. (2008)        |
| UV-B radiation     | <i>Momordica charantia</i> | Agarwal and Shaheen (2007)    |
|                    | <i>Picea asperata</i>      | Han et al. (2009)             |



**FIGURE 5.3** Antioxidant defense system comprises enzymatic and nonenzymatic components. These components, singly or in combination, can be successfully used as attractive targets to produce abiotic stress-tolerant plants using biotechnological approaches.

### 5.3.1.1 Ascorbate

Ascorbate is the most abundant low-molecular-weight antioxidant in plants and is proposed to function, along with other members of the antioxidant network, in controlling the level of ROS (Colville and Smirnoff, 2008). AsA has been shown to play an important role in several physiological processes in plants, including growth, differentiation, and metabolism. AsA has a key role in defense against oxidative stress and is particularly abundant in photosynthetic tissues (Smirnoff et al., 2004). It exists in the reduced state (AsA) as well as in oxidized forms (such as monodehydroascorbate; MDHA and dehydroascorbate; DHA) in plants. Most of AsA, almost more than 90%, is localized in cytoplasm, but unlike other soluble antioxidants, a substantial portion is exported to the apoplast, where it is present in millimolar concentration. Apoplastic AsA is believed to represent the first line of defense against potentially damaging external oxidants (Barnes et al., 2002). AsA protects critical macromolecules from oxidative damage.

AsA has a key role in the removal of  $\text{H}_2\text{O}_2$  via AsA–GSH cycle (Pinto et al., 2003). It also reacts directly with  $\text{O}_2^{\cdot-}$ ,  $\text{H}_2\text{O}_2$ , or the tocopheroxyl radical to form MDHA and DHA. The oxidation of AsA occurs in two sequential steps, first producing MDHA and subsequently DHA. The MDHA radical is also a primary product of APX reaction in the chloroplasts (Asada, 1997). The MDHA can either spontaneously dismutate or it gets reduced to AsA by NADP(H)-dependent enzyme, MDHAR (Miyake and Asada, 1994). If MDHA is not rapidly rereduced to AsA, the MDHA radical spontaneously disproportionates to AsA and DHA. DHA is also highly unstable at pH values greater than 6.0 and gets decomposed to tartarate and oxalate (Noctor and Foyer, 1998). To prevent this, DHA is rapidly reduced to AsA by the enzyme DHAR using reducing equivalents from GSH (Asada, 1996). AsA level has been reported to alter in response to drought (Sharma and Dubey, 2005; Esfandiari et al., 2008), salinity (Hernandez et al., 2001), chilling (Radyuk et al., 2009), metal toxicity (Sharma and Dubey, 2007; Maheshwari and Dubey, 2009), anaerobiosis (Lin et al., 2004), gaseous pollutants (Ranieri et al., 1997; Scebba et al., 2003; Severino et al., 2007), UV-B radiation (Agarwal and Shaheen, 2007), etc. The level of AsA under environmental stresses depends on the balance between the rates and the capacity of AsA biosynthesis and turnover related to antioxidant demand (Chaves et al., 2002). AsA concentration has a crucial role in the regulation of the compartmentalization of the

antioxidant system in *Arabidopsis*. In AsA deficient mutants of *Arabidopsis vtc-1*, total AsA in the apoplast decreased by nearly 23% of the wild-type value. Total leaf peroxidase activity increased in the mutants and compartment-specific differences in APX activity were observed. The activity and expression of cytosolic APX increased, whereas that for chloroplast APX, isoforms either remained unchanged or slightly decreased. Mutant studies have shown the importance of AsA in abiotic stress tolerance in plants. Low intrinsic AsA level in the *vtc-1* mutant under salt stress induced a dramatic decrease in the reduced form of AsA, which resulted in enhanced ROS contents in the *vtc-1* mutants (Huang et al., 2005). Similarly, AsA-deficient mutant *vtc-1* was found to be more sensitive to supplementary UV-B treatment than wild type plants (Gao and Zhang, 2008).

### 5.3.1.2 Glutathione

The reduced form of the tripeptide glutathione ( $\gamma$ -glutamyl-cysteinyl-glycine, GSH), is the major low-molecular-weight nonprotein thiol present in most plants. GSH is synthesized in the cytosol and chloroplasts of plant cells by compartment-specific isoforms of  $\gamma$ -glutamyl-cysteinyl synthetase ( $\gamma$ -ECS) and glutathione synthetase (GS). Besides serving as a key antioxidant within the cell, GSH acts as a redox buffer, is involved in the detoxification of xenobiotics, and it serves as a precursor for the synthesis of phytochelatins, which are crucial in controlling cellular heavy metal concentrations by chelating and sequestering them in vacuoles (Foyer et al., 2001). Due to its reducing power, GSH plays an important role in diverse biological processes in order to maintain cellular homeostasis, such as cell growth/division, metabolic reducing reactions, transmembrane transport, receptor actions, synthesis of proteins and nucleic acids, etc. (Foyer et al., 1997). GSH functions as an antioxidant in many ways. It can react chemically with  $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$  and, therefore, can function directly as a free radical scavenger. GSH can protect macromolecules (i.e., proteins, lipids, DNA) either by the formation of adducts directly with reactive electrophiles (glutathiolation) or by acting as proton donor in the presence of ROS or organic free radicals yielding glutathione disulfide (GSSG) (Asada, 1994). Under unstressed conditions, GSSG is reduced efficiently back to GSH by the action of enzyme GR present in cytosol, mitochondria, and chloroplasts. In extreme stress situations, the rate of GSH oxidation exceeds GSH reduction and the ratio of GSH/GSSG decreases (Foyer et al., 2001). GSH recycles AsA from its oxidized form to its reduced form by the enzyme DHAR (Loewus, 1988). GSH can also reduce DHA by a nonenzymic mechanism at pH > 7 and at GSH concentrations greater than 1 mM. This may be a significant pathway in chloroplasts, where in the presence of light, pH remains around 8 and GSH concentration may be as high as 5 mM (Foyer and Halliwell, 1976). The enzyme GR uses NADPH as a cofactor to reduce GSSG back to two molecules of GSH. The reductant GSH, together with its oxidized sulfur-sulfur linked form (GSSG), forms a very potent redox couple and is considered to be the major redox buffer of the cell. Oxidative stress results in the formation of GSSG at the expense of GSH. This shift in the ratio of GSH/GSSG would change the redox status to a less negative potential. If this potential rises too much, it would either stimulate or impede redox-sensitive important cellular processes like signal transduction pathways, calcium release, enzyme activation, etc. (Watanabe et al., 1972; Ernest and Kim, 1973). Therefore, the generation and maintenance of reduced GSH pool, either by de novo synthesis or via recycling by GR, using NADPH as a cofactor and electron donor, is of vital importance for the cell. The role of GSH in the antioxidative defense system provides a rationale for its use as a stress marker. A time-course analysis to monitor the level of GSH during stress response indicated that an initial stress response was related to changes in the GSH redox state, whereas acclimation was marked by increased GSH concentrations, increased GSH synthesizing enzyme activities, and/or a more reduced redox state of GSH (Tausz et al., 2004). The latter was interpreted as overcompensation, leading to the enhanced regeneration of GSH. Deteriorative effects on strong stress impacts were related to the progressive degradation and oxidation of the GSH pool. When apple trees were subjected to progressive drought, the initial response was a little oxidation of the GSH pool, followed by increased GSH concentrations. When the stress increased, GSH concentrations dropped and redox state became more oxidized, which marked the degradation of the system (Tausz et al., 2004). Similar to drought stress, the altered ratio of GSH/GSSG has been observed in plants under various stresses

like salinity (Hefny and Abdel-Kader, 2009), chilling (Radyuk et al., 2009), metal toxicity (Sharma and Dubey, 2007; Maheshwari and Dubey, 2009), anaerobiosis (Lin et al., 2004), and gaseous pollutants (Scebba et al., 2003). However, the importance of the GSH system relative to the other components of the antioxidative defense system, as well as relative to stress avoidance strategies, remains to be established (Tausz et al., 2004).

### 5.3.1.3 Tocopherol and Carotenoids

Like AsA and GSH, carotenoids and tocopherol are important antioxidants of the plant cell. Tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) represent a group of lipophilic antioxidants that are synthesized only by photosynthetic organisms. They scavenge oxygen free radicals, lipid peroxy radicals, and  $^1\text{O}_2$  (Diplock et al., 1989). Fully substituted benzoquinone ring and fully reduced phytyl chain of tocopherol act as antioxidants in redox interactions with  $^1\text{O}_2$  (Fryer, 1992; Halliwell and Gutteridge, 1999). Tocopherol also acts as a membrane stabilizer. It is widely believed that the protection of pigments and proteins of photosynthetic system and PUFA from oxidative damage caused by ROS is the main function of tocopherols. Mutants of *Arabidopsis thaliana* with T-DNA insertions in tocopherol biosynthesis genes, tocopherol cyclase (*vte1*), and  $\gamma$ -tocopherol methyltransferase (*vte4*) showed higher concentration of protein carbonyl groups and GSSG compared to the wild type, indicating the development of oxidative stress (Semchuk et al., 2009). The accumulation of  $\alpha$ -tocopherol has been shown to induce tolerance to chilling, water deficit and, salinity in different plant species (Yamaguchi-Shinozaki and Shinozaki, 1994; Munné-Bosch et al., 1999; Guo et al., 2006; Bafeel and Ibrahim, 2008).

Carotenoids function as accessory pigments in light harvesting during photosynthesis, but, perhaps, a more important role of carotenoids is their ability to detoxify the various forms of activated oxygen species (Young, 1991). Carotenoids also serve as precursors to signaling molecules that influence plant development and biotic/abiotic stress responses (Li et al., 2008). The ability of carotenoids to scavenge, prevent, or minimize the production of triplet chlorophyll may be accounted for by their chemical specificity. Carotenoids contain a chain of isoprenic residues bearing numerous conjugated double bonds, which allows easy energy uptake from excited molecules and dissipation of excess energy as heat (Edge et al., 1997; Mittler, 2002). Yildiz-Aktas et al. (2009) observed a higher content of carotenoids and antiradical capacity and lower MDA level in the drought-tolerant genotype of cotton compared with the sensitive genotype at normal water supply. In plants subjected to water deficit, a decline in the level of carotenoids and antiradical capacity was observed. However, this response was less pronounced in the tolerant than in the sensitive genotype; that is, despite the stress conditions imposed, the tolerant plants maintained a more effective defense system. Hence, carotenoids were correlated with drought tolerance in cotton plants (Yildiz-Aktas et al., 2009). The first committed step in the plastid-localized biosynthetic pathway of carotenoids is mediated by the nuclear-encoded phytoene synthase (PSY). PSY1 was found to have a role in heat stress tolerance (Li et al., 2008). Chromoplast carotenoids are known to accumulate in green tissues experiencing stress conditions, and studies indicate that they provide efficient protection against oxidative stress. Experiments conducted to test the role of ROS as the regulators of chromoplast carotenoid biosynthesis *in vivo*, revealed that the addition of ROS progenitors, such as menadione, *tert*-butylhydroperoxide, or paraquat and prooxidants like diamide or buthionine sulfoximine to green pericarp discs of pepper fruits, rapidly and dramatically induced the simultaneous expression of multiple carotenogenic gene-related mRNAs that could give rise to capsanthin. Similarly, down-regulation of CAT by amitrole induces the expression of carotenogenic gene-related mRNAs, leading to the synthesis of capsanthin in excised green pericarp discs. ROS signals from plastids and mitochondria were also shown to contribute significantly to this process (Bouvier et al., 1998).

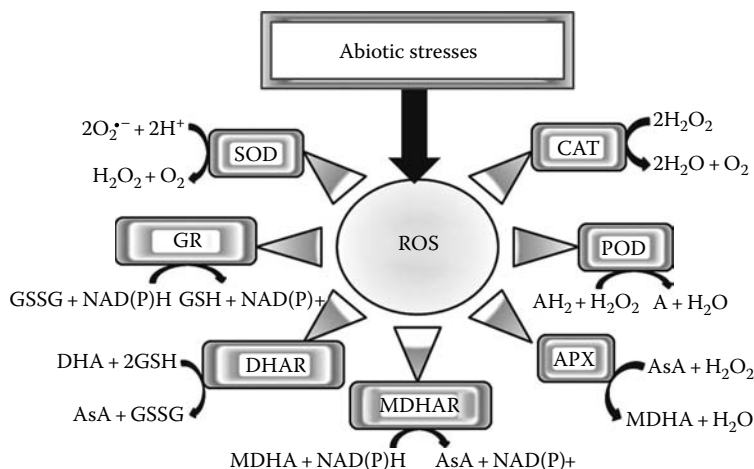
### 5.3.1.4 Phenolic Compounds

Besides the well-studied nonenzymic antioxidants like AsA, GSH, tocopherols, recent works have begun to highlight the potential role of flavonoids, phenylpropanoids, and phenolic acids as effective

antioxidants. Phenolics contain an aromatic ring with —OH or OCH<sub>3</sub> substituents that together contribute to their biological activity. There are two major phenolic classes, hydroxycinnamic acids and flavonoids. Flavonoids act as antioxidants and UV-B protectant. Most flavonoids outperform well-known antioxidants, AsA, and  $\alpha$ -tocopherol, in *in vitro* antioxidant assays because of their strong capacity to donate electrons or hydrogen atoms. However, the antioxidant function of flavonoids in plants is still a matter of debate (Hernandez et al., 2009). During heavy metal stress, phenolic compounds can act as metal chelators and on the other hand, phenolics can directly scavenge molecular species of active oxygen. Phenolics, especially flavonoids and phenylpropanoids, are oxidized by peroxidase, and act in H<sub>2</sub>O<sub>2</sub>-scavenging, phenolic/AsA/POD system. Their antioxidant action resides mainly in their chemical structure. There is some evidence of the induction of phenolic metabolism in plants as a response to multiple stresses (including heavy metal stress) (Michalak, 2006). When the influence of a high copper sulfate concentration on lipid peroxidation and the contents of total phenolic compounds in roots of lentil (*Lens culinaris* Medic.) cvs. Krak and Tina were investigated, it was observed that ROS could serve as a common signal for acclimation to Cu<sup>2+</sup> stress and could cause the accumulation of total phenolic compounds in dark-grown roots (Janas et al., 2009). It is believed that phenolics including various flavonoids serve as “sunscreens” to UV exposure: in particular, flavonoids play pivotal roles in absorbing free radicals, quenching <sup>1</sup>O<sub>2</sub>, and decomposing peroxides. A mutant *Arabidopsis thaliana* L., which displays a dramatic increase in sensitivity to UV-B radiation compared with wild-type plants, was found to have a single gene defect that led to a block in the synthesis of a group of flavonoids that normally accumulate in developing wild-type *Arabidopsis* and that increase in concentration when plants are exposed to UV radiation. One of these compounds has been identified as a rhamnosylated derivative of the flavonol, kaempferol. Therefore, it was suggested that flavonoids play an important role in the protection of plants from the damaging effects of UV-B light (Lois and Buchanan, 1994).

### 5.3.2 ENZYMATIC DEFENSE SYSTEM

The enzymatic components of the antioxidative defense system comprise several antioxidant enzymes such as SOD, CAT, GPX, enzymes of AsA–GSH cycle APX, MDHAR, DHAR, and GR (Noctor and Foyer, 1998). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. A detailed account of the reactions catalyzed by various antioxidant enzymes has been presented in Figure 5.4.



**FIGURE 5.4** Reactions catalyzed by enzymes involved in antioxidative defense system in plants. The efficient scavenging/destruction of ROS generated during abiotic stresses requires the concerted action of these antioxidant enzymes.



### 5.3.2.1 Superoxide Dismutase

The enzyme SOD belongs to the group of metalloenzymes and catalyzes the disproportionation of  $O_2^{\cdot-}$  to  $O_2$  and  $H_2O_2$ . Within the cell, SOD constitutes the first line of defense against ROS (Alscher et al., 2002). This enzyme neutralizes the very reactive  $O_2^{\cdot-}$  produced in the different compartments of plant cells into  $O_2$  and  $H_2O_2$  with the rate  $10^4$  times faster than the spontaneous dismutation reaction (Fridovich, 1975). Since SOD is present in all aerobic organisms and in most of the sub-cellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in defense against oxidative stress (Scandalios, 1993). While all compartments of the cell are possible sites for  $O_2^{\cdot-}$  formation, chloroplasts, mitochondria, and peroxisomes are thought to be the most important generators of ROS (Alscher et al., 2002). In plant systems, three isozymes of SOD, namely, Cu/Zn-SOD, Mn-SOD, Fe-SOD have been reported (Fridovich, 1989; Racchi et al., 2001). All forms of SOD are nuclear encoded and targeted to their respective subcellular compartments by an amino terminal targeting sequence (Bowler et al., 1992). Cu/Zn-SOD is predominantly found in cytosol, chloroplast, and mitochondria, Mn-SOD in mitochondria and peroxisomes, whereas Fe-SOD is predominantly present in chloroplasts (del Rio et al., 1998; Arora et al., 2002). Cu/Zn-SOD is cyanide sensitive whereas the other two (Mn-SOD and Fe-SOD) are cyanide insensitive (del Rio et al., 1998). Eukaryotic Cu/Zn-SOD isoforms are dimers whereas Mn-SOD isoforms of mitochondria are tetramers (Scandalios, 1993).

The activity of SOD has been reported to increase under diverse stress situations such as drought, salinity, heat, chilling, metal toxicity, anaerobiosis, exposure to ozone, UV-B, etc. (Bowler et al., 1992; Rao et al., 1996; Lee and Lee, 2000; Verma and Dubey, 2003; Sharma and Dubey, 2005, 2007; Almeselmani et al., 2006; Yin et al., 2008; Han et al., 2009; Hefny and Abdel-Kader, 2009; Kumutha et al., 2009; Maheshwari and Dubey, 2009), and the increased activity is often correlated with the tolerance of the plant against these stresses. Decreased level of SOD was observed in drought-sensitive durum wheat landraces, whereas in resistant and moderately resistant durum wheat landraces, SOD remained unchanged or increased. Therefore, it was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials (Zaefyzadeh et al., 2009). The overproduction of SOD has been reported to result in enhanced oxidative stress tolerance in many plant species (McKersie et al., 1993; Perl et al., 1993; Foyer et al., 1994; Slooten et al., 1995a). Chary et al. (1994) constructed and characterized *Neurospora crassa* mutants that were null for *sod-1*, the gene that encodes Cu/Zn-SOD. Mutant strains were sensitive to elevated oxygen concentrations, and they exhibited an increased spontaneous mutation rate. The role of Mn-SOD in providing protection against ROS has been studied using Mn-SOD gene disruption. The nuclear gene for Mn-SOD of yeast mitochondria was inactivated by gene disruption. In the absence of oxygen, the mutant grew as rapidly as the wild-type parent. However, increasing concentrations of oxygen led to a progressive inhibition of growth. The properties of this mutant provide direct evidence that Mn-SOD contributes to the natural protection of cells against oxygen toxicity (van Loon et al., 1986). Similar to Cu/Zn-SOD and Mn-SOD, Fe-SOD has also been shown to play an important role in oxidative stress tolerance in plants. The transgenic *A. thaliana* line with high Fe-SOD activities showed enhanced tolerance to oxidative stress and showed increased growth rates compared to wild-type plants (Van Breusegem et al., 1999).

### 5.3.2.2 Catalase

Catalase is a tetrameric, heme-containing enzyme found in all aerobic organisms and catalyzes the dismutation of  $H_2O_2$  into water and oxygen.  $H_2O_2$  has been implicated in many stress conditions. In plants, CAT scavenges  $H_2O_2$  generated during mitochondrial electron transport,  $\beta$ -oxidation of fatty acids, and, most importantly, during photorespiratory oxidation (Scandalios et al., 1997). CAT is unique among  $H_2O_2$  degrading enzymes as it degrades  $H_2O_2$  without consuming cellular reducing equivalents. Therefore, when cells are stressed for energy and are rapidly generating  $H_2O_2$  through catabolic processes,  $H_2O_2$  is degraded by CAT in an energy efficient manner (Mallick and Mohn, 2000).

Different classes of CAT have been identified based on their expression profile. A nomenclature has been adopted for this classification (Willekens et al., 1994). Class I CATs are expressed in photosynthetic tissues and are regulated by light. Class II CATs are expressed at high levels in vascular tissues, whereas class III CATs are highly abundant in seeds and young seedlings (Willekens et al., 1994). CATs are very sensitive to light and have very rapid turnover rate similar to that of D1 protein of PS II (Hertwig et al., 1992). This may be as a result of light absorption by the heme group or perhaps inactivation due to  $\text{H}_2\text{O}_2$ . Abiotic stresses cause either the enhancement or depletion of CAT activity (Feierabend et al., 1992; Fu and Huang, 2001; El-Shintinawy et al., 2004; Sharma and Dubey, 2005; Moussa and Abdel-Aziz, 2008; Han et al., 2009; Kumutha et al., 2009; Noreen and Ashraf, 2009). The properties of CATs suggest that the enzyme is inefficient in removing low concentration of  $\text{H}_2\text{O}_2$  (Arora et al., 2002).

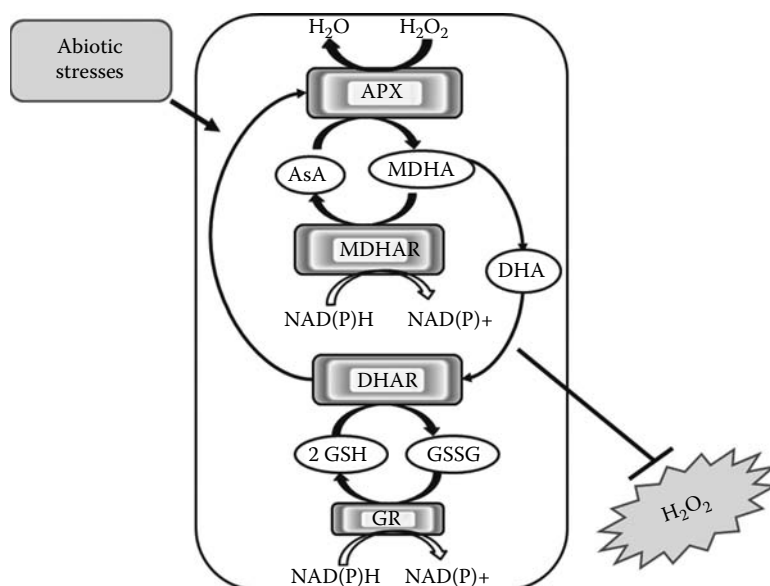
CATs function as a cellular sink for the removal of  $\text{H}_2\text{O}_2$ , as evidenced by the complementation of CAT deficiency by the exogenous application of CAT, and comparison of CAT-deficient and control leaf discs in removing external  $\text{H}_2\text{O}_2$ . Stress analysis revealed increased susceptibility of CAT-deficient plants to paraquat, salt, and ozone, but not to chilling (Willekens et al., 1997). Transgenic tobacco plants having 10% wild-type CAT activity showed no visible disorders at low light, but in elevated light, rapidly developed white necrotic lesions on the leaves. Leaf necrosis in such plants was correlated with the accumulation of GSSG and a fourfold decrease in AsA, indicating that CAT is critical for maintaining the redox balance during oxidative stress (Willekens et al., 1997). Palatnik et al. (2002), while investigating the antioxidant status in a CAT-deficient mutant of barley RPr79/4, and in its motherline cv. Maris Mink, observed that seedlings of the CAT-deficient mutant grown in a growth chamber under a 14-h photoperiod ( $200 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ), showed higher concentrations of GSH and APX as compared to wild-type plants. An additional mitochondrial Mn-SOD isoenzyme was also detected in RPr79/4. When seedlings of the CAT-deficient mutant were grown at higher light intensities ( $370 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ), a Cu/ZnSOD isoform and the cytosolic glutamine synthetase isoenzyme were concomitantly induced. Taken together, these results suggest that several defense mechanisms operating in different subcellular compartments respond in concert to compensate for CAT deficiency in barley seedlings exposed to oxidative stress (Palatnik et al., 2002).

### 5.3.2.3 Guaiacol Peroxidase

Peroxidases (PODs) belong to a large family of enzymes that occur ubiquitously in fungi, plants, and vertebrates and convert  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$  and  $\text{O}_2$  (Mika and Luthje, 2003). Classical secretory plant PODs that commonly use guaiacol as a reducing substrate are also referred to as guaiacol peroxidase (GPX). These proteins usually contain a ferri protoporphyrin IX prosthetic group that oxidizes several substrates in the presence of  $\text{H}_2\text{O}_2$  (Vianello et al., 1997). Most secretory plant PODs are glycosylated (Johansson et al., 1992). These enzymes have four conserved disulfide bridges and contain two structural  $\text{Ca}^{2+}$  ions (Schuller et al., 1996). Many isoenzymes of GPX exist in plant tissues localized in vacuoles, the cell wall, and the cytosol (Asada, 1992). GPX oxidizes a wide range of organic substrates, and the oxidation processes are associated with many important biosynthetic processes. GPXs have been proposed to participate in the lignification of cell wall, degradation of IAA, biosynthesis of ethylene, wound healing, and defense against pathogens (Kobayashi et al., 1996). Both intracellular and extracellular PODs have an important role in the antioxidative response of plant cells (Siegel and Siegel, 1986). PODs can function as effective quenchers of reactive intermediary forms of  $\text{O}_2$  and peroxy radicals under stressed conditions (Vangronsveld and Clijsters, 1994). Various stressful conditions of the environment have been shown to induce the activity of PODs (Rao et al., 1996; Radotic et al., 2000; Shah et al., 2001; El-baky et al., 2003; Harinasut et al., 2003; Verma and Dubey, 2003; Sharma and Dubey, 2005, 2007; Moussa and Abdel-Aziz, 2008; Han et al., 2009; Maheshwari and Dubey, 2009). PODs have been used as biochemical markers for various types of abiotic and biotic stresses (Castillo et al., 1992). Radotic et al. (2000) correlated the increased activity of PODs to oxidative reactions under metal toxicity conditions and suggested its potential as a biomarker for sublethal metal toxicity in plants.

### 5.3.2.4 Enzymes of Ascorbate–Glutathione Cycle

Efficient scavenging/destruction of ROS generated during abiotic stresses require the action of several antioxidant enzymes. The AsA–GSH cycle, also referred to as Halliwell–Asada pathway, present in at least four different subcellular locations including the cytosol, chloroplast, mitochondria, and peroxisomes, scavenges  $\text{H}_2\text{O}_2$ . The AsA–GSH cycle involves successive oxidation and reduction of AsA, GSH, and NADPH catalyzed by the enzymes APX, MDHAR, DHAR, and GR (Figure 5.5). APX uses two molecules of AsA to reduce  $\text{H}_2\text{O}_2$  to water with a concomitant generation of two molecules of MDHA. APX is a member of Class I super family of heme PODs (Welinder, 1992). In higher plants, five chemically and enzymatically distinct isoenzymes of APX have been found differing in their subcellular localization and amino acid sequences. These are cytosolic, stromal, thylakoidal, mitochondrial, and the peroxisomal isoforms (Jimenez et al., 1997). The classification is based on sequence comparisons rather than on function. APX isoenzymes also differ in their molecular weight, substrate specificity, pH optima, and stability (Wang et al., 1999). Cytosolic APX has been characterized in photosynthetic as well as nonphotosynthetic tissues (Madhusudhan et al., 2003). The chloroplastic and cytosolic APX isoforms are specific for AsA as electron donor and the cytosolic isoenzymes are less sensitive to depletion of AsA than the chloroplastic isoenzymes including stromal- and thylakoid-bound enzymes (Ishikawa et al., 1998). Many workers have reported enhanced expression of APX in response to abiotic stresses such as drought, salinity, heat, chilling, metal toxicity, anaerobiosis, UV irradiation, gaseous pollutants, etc. (Rao et al., 1996; Boo and Jung, 1999; Sharma and Dubey, 2005, 2007; Han et al., 2009; Hefny and Abdel-Kader, 2009; Kumutha et al., 2009; Locato et al., 2009; Maheshwari and Dubey, 2009; Radyuk et al., 2009). The importance of APX in stress defense was demonstrated in APX-antisense transgenic tobacco that was highly susceptible to ozone injury compared to the wild-type plants (Orvar and Ellis, 1997). The overexpression of a cytosolic APX gene derived from pea (*Pisum sativum* L.) in transgenic tomato plants (*Lycopersicon esculentum* L.) resulted in lower electrolyte leakage (20%–23%) than wild type (44%) after exposure to 4°C. The visual assessment of transgenic



**FIGURE 5.5** Components of ascorbate–glutathione cycle play an important role in the decomposition of  $\text{H}_2\text{O}_2$  in plants under abiotic stresses. APX reduces  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  using AsA, which generates MDHA. MDHA can be reduced to AsA by MDHAR. If not reduced rapidly, MDHA is disproportionated into AsA and DHA. DHA is reduced to AsA by DHAR using GSH as the reducing agent. GSH gets converted to GSSG that, in turn, is reduced by GR using NADPH.

and wild-type lines exposed to salinity stress (200 or 250 mM) confirmed that the overexpression of APX minimized leaf damage. Moreover, APX activity was nearly 25- and 10-fold higher in the leaves of transgenic plants in response to chilling and salt stresses, respectively. Results substantiate that increased levels of APX activity brought about by the overexpression of a cytosolic APX gene may play an important role in ameliorating oxidative injury induced by chilling and salt stress (Wang et al., 2005b).

In plant cells, AsA is a major antioxidant that is part of the AsA–GSH cycle. MDHAR, the enzymatic component of this cycle, is involved in the regeneration of reduced AsA. MDHA radical produced in APX catalyzed reaction has a short lifetime and if not rapidly reduced, it disproportionates to AsA and DHA (Ushimaru et al., 1997). Within the cell, such as at the plasmalemma or at the thylakoid membrane, MDHA can be reduced directly to AsA. The electron donor for MDHA reduction may be b-type cytochrome, reduced ferredoxin, or NAD(P)H. The reaction is catalyzed by the enzyme MDHAR, which is found in several cellular compartments (Miyake and Asada, 1994). The isoenzymes of MDHAR have been reported to be present both inside and outside the plastids (De Leonardis et al., 1995) and in shoots, roots, dry seeds, as well as fruits (Ushimaru et al., 1997; Leterrier et al., 2005). Various research groups have shown increased activity of MDHAR in plants subjected to abiotic stresses (Boo and Jung, 1999; Sharma and Dubey, 2005, 2007; Locato et al., 2009; Maheshwari and Dubey, 2009). The presence of different regulatory motifs in the promoter region of the *MDAR1* gene has been suggested to be responsible for the distinct responses of plants to various stress conditions. The functional analysis of MDHAR isoforms present in the different cell compartments in pea plants grown under eight stress conditions, including continuous light, high light intensity, continuous dark, mechanical wounding, low and high temperatures, excess Cd, and under the application of the herbicide 2,4-dichlorophenoxyacetic acid revealed a significant induction of MDAR activity by high light intensity and Cd. On the other hand, expression studies demonstrated differential expression patterns of peroxisomal *MDAR 1* transcript in pea plants grown under these stressful conditions. These findings show that the peroxisomal *MDAR 1* has a differential regulation that could be indicative of its specific function in peroxisomes (Leterrier et al., 2005).

Despite the possibility of enzymic and nonenzymic regeneration of AsA directly from MDHA, some DHA is always produced when AsA is oxidized in leaves and other tissues. DHA is reduced to AsA by the action of DHAR using GSH as the reducing substrate (Ushimaru et al., 1997). DHAR is a key component of the AsA recycling system. DHAR is a monomeric thiol enzyme abundantly found in dry seeds, roots, and etiolated as well as green shoots. Three functional DHAR genes have been reported to be encoded in the *Arabidopsis* genome. Abiotic stresses such as drought, metal toxicity, chilling, ozone exposure etc., increase the activity of the DHAR in plants (Boo and Jung, 1999; Hernandez et al., 2001; Sharma and Dubey, 2005, 2007; Yoshida et al., 2006; Locato et al., 2009; Maheshwari and Dubey, 2009). An *Arabidopsis* mutant completely lacking cytosolic DHAR activity was found to be highly ozone sensitive. The amounts of total AsA and GSH were similar in both lines, but the amount of apoplastic AsA in the mutant was 61.5% lower. These results indicate that the apoplastic AsA, which is generated through the reduction of DHA by cytosolic DHAR, is important for ozone tolerance (Yoshida et al., 2006). Consistent upregulation of the gene encoding cytosolic DHAR was found in *L. japonicas*, which was found to be more tolerant to salt stress than other legumes. This upregulation of DHAR was correlated to its role in AsA recycling in the apoplast (Rubio et al., 2009).

Reaction catalyzed by DHAR generates GSSG that in turn gets rereduced to GSH using NADPH in a reaction catalyzed by enzyme GR. GR is a NAD(P)H-dependent enzyme ubiquitously present in mesophyll cells. Although it is located in chloroplasts, cytosol, and mitochondria, around 80% of GR activity in photosynthetic tissues is accounted for by chloroplastic isoforms (Edwards et al., 1990). GR has been purified and well characterized from the leaves of several plant species such as spinach (Halliwell and Foyer, 1978), pea (Kalt-Torres et al., 1984), and corn (Mahan and Burke, 1987). In most of the plants, GRs are homodimers with a molecular mass ranging from 100 to 150 kDa, containing one FAD per monomer. GR belongs to a group of flavoenzymes including lipoyl

dehydrogenase, thioredoxin reductase, tripanetine reductase, and mercuric reductase, and contain an essential disulfide group (Ghisla and Massey, 1989). The catalytic mechanism involves two steps: first, the flavin moiety is reduced by NADPH, the flavin is oxidized and a redox-active disulfide bridge is reduced to produce a thiolate anion and a cysteine. The second step involves the reduction of GSSG *via* thiol–disulfide interchange reactions (Ghisla and Massey, 1989). If the reduced enzyme is not reoxidized by GSSG, it can suffer a reversible inactivation. Several authors have reported the increased activity of this enzyme under abiotic stresses (Rao et al., 1996; Hernandez et al., 2001; Sharma and Dubey, 2005, 2007; Yoshida et al., 2006; Kumutha et al., 2009; Locato et al., 2009; Maheshwari and Dubey, 2009). Pastori and Trippi (1992) observed a correlation between the oxidative stress resistance and the activity of GR and suggested that oxidative stress caused by paraquat or  $\text{H}_2\text{O}_2$  could stimulate GR *de novo* synthesis, probably at the level of translation by pre-existing mRNA.

## 5.4 LEVEL OF ROS, EXTENT OF OXIDATIVE STRESS, AND THE STATUS OF ANTIOXIDATIVE DEFENSE SYSTEM UNDER VARIOUS ABIOTIC STRESSES

Oxidative stress is one of the major limiting factors in crop productivity. Various stressful conditions of the environment such as drought, salinity, extreme temperatures, excessive levels of metals in the soil, anaerobiosis, gaseous pollutants, UV-B radiation, etc., affect the physiological processes of plants and lead to the production of ROS that in turn cause oxidative damage at the cellular level (Radotic et al., 2000; Abarca et al., 2001). Active oxygen species must be rapidly processed if oxidative damage is to be averted. An efficient antioxidative defense system can scavenge overproduced ROS in plants. The lifetime of ROS within the cellular environment is determined by the efficiency of the antioxidative system (both nonenzymatic and enzymatic antioxidants), which provides crucial protection against oxidative damage (Noctor and Foyer, 1998). The present section describes the status of the production of ROS, the induction of oxidative stress, and the roles of individual components of the antioxidative defense system under various abiotic stresses.

### 5.4.1 DROUGHT

Among the stressful conditions of the environment, drought serves as major constraint, limiting crop production in arid and semiarid areas of the world. Growth and the primary production of plants are severely reduced by water deficit (Tambussi et al., 2000; Fatima et al., 2005). The inhibition of carbon dioxide ( $\text{CO}_2$ ) assimilation, coupled with the changes in photosystem activities, and photosynthetic transport capacity under drought stress result in the accelerated production of ROS *via* the chloroplast Mehler reaction (Asada, 1999). ROS enhancement under drought stress functions as an alarm signal that triggers acclimatory/defense responses by specific signal transduction pathways that involve  $\text{H}_2\text{O}_2$  as secondary messenger. ROS signaling is linked to abscisic acid (ABA),  $\text{Ca}^{2+}$  fluxes, and sugar sensing, and is likely to be involved both upstream and downstream of the ABA-dependent signaling pathways under drought stress. Nevertheless, if drought stress is prolonged above a certain extent, ROS production will overwhelm the scavenging action of the antioxidant system, resulting in extensive cellular damage and death (de Carvalho, 2008). Root is the first plant organ to sense reduced water supply. Signals are generally sent from roots to the leaves through the xylem sap, and the phytohormone abscisic acid is considered to be one of the major root-to-shoot stress signals. After reaching leaves, stress signals trigger stomatal closure and the plant shifts to a water-saving strategy. By adjusting stomatal opening, plants control water loss by reducing the transpiration flux, but the entrance of  $\text{CO}_2$  is also reduced concomitantly. Limited supply of  $\text{CO}_2$  to chloroplasts causes lack of electron acceptors. As a result, the electron transport chain is over reduced and the production of ROS increases. The limitation on  $\text{CO}_2$  fixation reduces  $\text{NADP}^+$  regeneration, leading to an over reduction of the photosynthetic electron transport chain. Thus, during water deficit, there is a considerable potential

for increased accumulation of  $O_2^{\cdot-}$  and  $H_2O_2$  resulting from the increased rate of  $O_2$  photoreduction in chloroplasts (Robinson and Bunce, 2000). A higher leakage of electrons to  $O_2$  by the Mehler reaction has been observed in plants subjected to drought stress. Biehler and Fock (1996) reported 50% more leakage of photosynthetic electrons to the Mehler reaction in drought-stressed wheat plants, compared to unstressed plants. Increased formation of ROS, lipid peroxidation, and protein modification has been observed in several plant species subjected to drought stress (Boo and Jung, 1999; Sharma and Dubey, 2005; Bai et al., 2006; Esfandiari et al., 2008). The sequence of events in plant tissues subjected to drought include (1) increased production of ROS and of oxidized target molecules, (2) increase in the expression of genes for antioxidant functions, (3) increase in the levels of nonenzymic and enzymic antioxidants etc., (4) resulting in the tolerance against drought stress (Mano et al., 2002). Drought stress increases the specific activity of some of the antioxidant enzymes and also induces the synthesis of new isoenzymes to overcome the increased oxidative stress (Srivalli et al., 2003; Sharma and Dubey, 2005). Enhanced activity of enzymes of antioxidative defense system has been reported under drought stress in *Zea mays* (Jagtap and Bhargava, 1995), *Oryza sativa* (Boo and Jung, 1999; Sharma and Dubey, 2005), *Triticum durum* (Sgherri et al., 2000), and *Camellia sinensis* (Rajamickam et al., 2005) plants. The degree to which the activities of antioxidant enzymes and the amount of antioxidants increase in drought-stressed plants appears to be extremely variable among several plant species, even between different cvs. of the same species. Reddy et al. (2004) observed that among five mulberries (*Morus alba* L.) cultivars (BC2-59, K-2, MR-2, S-13, and TR-10) subjected to drought, cvs. S-13 and BC2-59 had efficient antioxidative characteristics that could provide better protection against oxidative stress in leaves under water-limited conditions. Compared to drought-sensitive maize genotype (Trihybrid 321), the drought-tolerant maize genotype (Giza 2) exhibited lower accumulation of MDA and  $H_2O_2$  content and increased activities of antioxidant enzymes SOD, CAT, and POD under water stress (Moussa and Abdel-Aziz, 2008). Differential responses for antioxidative enzymes SOD and POD to progressive drought stress were also found between the two contrasting populations (wet climate and dry climate population) of *Populus cathayana* Rehder. One of the reasons for difference in drought tolerance was suggested to be the increased capacity of the antioxidative system to scavenge ROS, and the consequent suppressed level of lipid peroxidation under drought conditions (Xiao et al., 2008a). The response of a plant species to drought not only depends on the species inherent “strategy” but also on the duration and severity of the drought period. Activities of SOD and CAT increased in response to severe water deficit in mature leaves of *Populus deltoides* × *nigra* clones, “Luisa Avanzo,” and “Dorskamp.” For both clones, three different SOD isoforms, Mn-SOD, Fe-SOD, and Cu/Zn-SOD were detected in varying amounts depending on drought intensity (Marron et al., 2006). An increase in the amount of AsA and GSH was observed as the level of drought stress was intensified in two widely cultivated cvs. of wheat in Iran, Sab., and N. Sar. (Esfandiari et al., 2008). In rice plants, increase in the capacity of AsA regeneration system by *de novo* synthesis of MDHAR, DHAR, and GR has been shown to be one of the primary responses to water deficit so as to mitigate oxidative stress (Boo and Jung, 1999; Sharma and Dubey, 2005). APX serves as an important component of antioxidative defense system under drought (Sharma and Dubey, 2005).

Al-Ghamdi (2009) showed the ability of wheat plants to acclimatize and induce the antioxidant defense system under drought stress. The drought-acclimated leaves exhibited systematic increase in the activity of  $H_2O_2$ -scavenging enzymes, particularly APX and CAT, and maintained adequate AsA redox pool by the efficient functioning of APX enzyme. As a result, lower membrane injury and a lower MDA content was observed in drought-acclimated plants (Al-Ghamdi, 2009).

#### 5.4.2 SALINITY

Soil salinity is one of the most serious constraints to crop productivity, especially in the arid and semiarid regions of the world. High concentrations of soluble salts in the soil environment cause nutrient imbalance, water deficit, and toxicity of salt ions in growing plants. Therefore, plants growing in saline soils have to encounter two types of stresses, osmotic stress and ion toxicity

(Lin and Kao, 2001; Munns, 2002). High salt concentrations normally impair the cellular electron transport and lead to the overproduction of the ROS- $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$ , and  $^1O_2$ . Several reports have demonstrated that salinity stress results in an excessive generation of these ROS (Tanou et al., 2009). Some researchers suggest that stomatal closure upon salt stress may limit the entry of  $CO_2$ , which, in turn, may cause the over reduction of photosynthetic electron transport system. Elevated  $CO_2$  mitigates the oxidative stress caused by salinity, involving lower ROS generation and a better maintenance of redox homeostasis as a consequence of higher assimilation rates and lower photorespiration (Perez-Lopez et al., 2009). NaCl-induced production of  $H_2O_2$  was likely linked to NAD(P)H-oxidase and amine oxidase regulation and was suggested to be signaled by nitric oxide (NO), salicylic acid (SA), protein kinase, and  $Ca^{2+}$  channel activity (Tanou et al., 2009). Enhanced production of ROS under salinity stress induces phytotoxic reactions such as lipid peroxidation, protein degradation, and DNA mutation (Hefny and Abdel-Kader, 2009; Tanou et al., 2009). The effects of salinity on cellular oxidative metabolism were analyzed in strawberry (*Fragaria x ananassa* Duch., cv. Selva) leaves. It was found that NaCl induced oxidative stress in strawberry leaves, as evidenced by an  $H_2O_2/O_2^{\cdot-}$  accumulation, an increase in lipid peroxidation and carbonyl groups content. NaCl caused the oxidation of AsA and GSH redox pairs and inhibition in the activities of some ROS-metabolizing enzymes including CAT, APX, GR (Tanou et al., 2009). In olive plants (*Olea europaea*), salinity was shown to induce NADPH-producing dehydrogenases in order to recycle NADPH necessary for the protection against oxidative damages. These NADP-dehydrogenases appear to be key antioxidative enzymes in olive plants under salt stress conditions (Valderrama et al., 2006).

The effect of salt stress depends on the organ of the plant, developmental stage of the plant, genotypes of the plant species, as well as the intensity and duration of the stress. Pronounced organ specificity of antioxidant defense system functioning was observed in *Plantago major* L. subjected to NaCl. The roots were characterized by high constitutive activities of SOD and three forms of peroxidase, and a lower CAT activity. Unlike leaves, the roots of *P. major* under salinity conditions possessed a higher activity of the antioxidant system, protecting plants from injurious action of oxidative stress, thereby providing the survival of this plant species under stress conditions (Radyukina et al., 2009). Salinity levels of 50 and 100 mM NaCl induced significant increase in SOD activity, GSH levels, and carotenoid concentrations in all tolerant genotypes and the local genotype of forage sorghum seedlings compared to sensitive group. It was suggested that these antioxidants can be considered as selection criteria for salt tolerance in sorghum species (Hefny and Abdel-Kader, 2009). Antioxidant systems depend on cultivars and cropping seasons. The antioxidant system in salt-stressed summer crop cultivars of "House Momotaro" was attributed to the enzymatic reactions of APX and GR, while in salt-stressed "Mini Carol" crop, it was attributed to the nonenzymatic reactions of AsA and GSH. In the winter crop, the antioxidant systems were not influenced by salt stress in either cultivar. The seasonal- and cultivar-specific differences of salt-induced changes in the antioxidant systems may result from differences in antioxidant capacities and the interaction between salt stress and growth conditions such as temperature and solar radiation (Zushi and Matsuzoe, 2009).

The changes in the leaf apoplastic antioxidant defenses in response to NaCl stress has been studied in two pea (*Pisum sativum*) cvs. Lincoln and Puget showed the different degrees of sensitivity to high NaCl concentrations. The results showed that SOD, AsA, GSH and, probably, DHAR were present in the leaf apoplastic space, whereas APX, MDHAR, and GR seemed to be absent. The AsA/DHA and GSH/GSSG ratios decreased progressively with the severity of the stress. Apoplastic SOD activity was induced in NaCl-treated pea cv. Puget but decreased in NaCl-treated pea cv. Lincoln. An increase in DHAR and GR and a decrease in APX, MDHAR, AsA, and GSH levels was observed in the symplast from NaCl-treated pea cv. Lincoln, whereas in pea cv. Puget, an increase in DHAR, GR, and MDHAR occurred. These results suggested a strong interaction between both cell compartments in the control of the apoplastic AsA content in pea leaves. Both the different antioxidative capacity and the NaCl-induced response in the apoplast and in the symplast from pea cv. Puget in comparison with pea cv. Lincoln was suggested to contribute to a better protection of pea cv. Puget against salt stress (Hernandez et al., 2001).

Using DNA microarray, Kawasaki et al. (2001) showed the salt-induced upregulation of several antioxidant genes in rice roots. Studies suggest that CAT, POD, and SOD isoenzymes can serve as useful markers in the analysis of gene functions and metabolic regulations, including salt-tolerance characteristics (Mittal and Dubey, 1991; Piqueras et al., 1996; El-baky et al., 2003). The specific activities and patterns of CAT, POD, and SOD isoenzymes are altered significantly in plants subjected to salinity stress. The induction of a new SOD isozyme was found in salt-tolerant embryonic callus cultures of lemon (*Citrus limon* L. Burn) (Piqueras et al., 1996). Rice seedlings, differing in salt tolerance, possess constitutively different number of peroxidase isoforms in nonsalinized seedlings. When these seedlings were raised under NaCl salinity, certain new isoforms of peroxidases appeared, and the intensities of some of the preexisting isoenzymes increased. In 15-day-old seedlings of a salt-tolerant rice cv. CSR-1, three isoenzymes were observed in roots and five in shoots, whereas in a salt-sensitive cv. Ratna, six isoenzymes were observed in the roots as well as in shoots (Mittal and Dubey, 1991). Similarly, electrophoretic bands of POD and CAT isoenzymes of onion cultivars were found to vary in numbers and relative concentrations due to salts stress. New protein bands and the characteristic CAT and POD isoenzymes banding patterns have been suggested as a biochemical marker for the selection of salt-tolerant plants (El-baky et al., 2003).

Changes in the activities of antioxidant enzymes and the levels of some nonenzymatic antioxidants were assessed for their use as markers of salt tolerance in nine genetically diverse pea (*Pisum sativum*) cvs. Salt stress markedly enhanced the activities of SOD and POD, as well as the levels of total phenolics and  $\gamma$ - and  $\delta$ -tocopherols, and decreased the total soluble proteins and CAT activity, while the internal levels of  $H_2O_2$  remained unaffected in all pea cvs. Of different antioxidant enzymes and metabolites analyzed, only CAT activity was found to be a reliable marker of salt tolerance in pea cultivars examined (Noreen and Ashraf, 2009).

The tolerance of *Physcomitrella patens* for high-salinity environments makes it an ideal candidate for studying the molecular mechanisms by which plants respond to salinity stresses. Differential genomic and proteomic screenings carried out in these plants showed that plants responded to salinity stress by upregulating a large number of genes involved in antioxidant defense mechanism (Wang et al., 2008), suggesting that the antioxidative system may play a crucial role in protecting cells from oxidative damage following exposure to salinity stress in *P. patens*.

### 5.4.3 HEAT

High temperature is becoming one of the significant abiotic stresses limiting plant growth and productivity, especially as the global temperature is likely to increase by 1.5°C–4.5°C by 2050 (Houghton et al., 2001). The damage to plants exposed to heat stress has been ascribed to the inhibition of photosynthesis, damage to cell membrane, senescence, and cell death (Xu et al., 2006). ROS could play a key role in mediating important signal transduction events. The rates and cellular sites of ROS production during high temperature or heat stress could play a central role in stress perception and protection. ROS levels, as well as ROS signals, are thought to be controlled by the ROS gene network of plants. It is likely that in plants, this network is interlinked with the different networks that control heat stress acclimation and tolerance. Suzuki and Mittler (2006) propose a model for the involvement of ROS in heat stress sensing and defense. Heat stress leads to the overproduction of ROS in the cells (Bukhov et al., 1999; Yin et al., 2008). Ribulose biphosphate carboxylase/oxygenase (Rubisco) can lead to the production of  $H_2O_2$  as a result of its oxygenase reactions and such  $H_2O_2$  production substantially with temperature (Kim and Portis, 2004). A considerable amount of work has revealed high temperature-induced oxidative damages in plants (El-Shintinawy et al., 2004; Yin et al., 2008). Lipid peroxidation level was directly correlated with temperature and exposure time in the seedlings of two Egyptian cultivars of barley (Giza 124 and 125). Heat shock caused an increase in the electrical conductivity of cell membrane and increased MDA content coupled with the disappearance of the polyunsaturated linolenic acid ( $C_{18:3}$ ) in the seedlings, reflecting the peroxidation of membrane lipids, which led to the loss of membrane selective



permeability (El-Shintinawy et al., 2004). Plant survival under heat stress requires the activation of proper defense mechanisms to avoid oxidative stress. Wheat genotypes HD 2815 and HDR-77 showed relatively higher SOD, APX, GR, CAT, and POX activity compared to PBW 343, PBW 175, and HD 2865 under high temperature stress. Genotypes showing highest activity of various antioxidant enzymes also showed minimum reduction in chlorophyll content and lower membrane injury index, indicating the amelioration of high temperature stress-induced oxidative stress by antioxidant enzymes (Almeselmani et al., 2006).

The antioxidant response depends on the severity and duration of the stress. When the effects of high temperature on antioxidant enzymes were investigated in three mulberry (*Morus alba* L.) cvs. K-2, MR-2, and BC2-59 maintained at 40°C for 120, 240, and 360 min., the activities of SOD, CAT, POD, APX, and GR increased with the duration of heat stress in all three cultivars. Cultivar BC2-59 showed efficient antioxidant system among the three cultivars, which could prevent the oxidative damage in the leaves caused due to high temperature stress (Chaitanya et al., 2002). El-Shintinawy et al. (2004) showed heat-induced distinct and significant changes in activities of antioxidant enzymes in barley. SOD and POD activities were progressively enhanced by moderate and elevated heat doses, but the most elevated one (45°C for 8 h) showed a decrease in activities of both enzymes. In contrast, CAT activity was reduced with all heat shocks (El-Shintinawy et al., 2004). In lily (*Lilium longiflorum* L.) plants, exposure to 37°C and 42°C for 10 h caused stimulation in the activities of antioxidant enzymes SOD, POD, CAT, APX, and GR and elevated levels of AsA and GSH, which resulted in low levels of  $O_2^{\cdot-}$  and  $H_2O_2$  concentrations. However, after 10 h exposure at 47°C, activities of SOD, APX, and GR, as well as the concentration of GSH were similar to the controls, while activities of POD, CAT and AsA concentration declined significantly as compared to the control, with a concomitant increase in  $O_2^{\cdot-}$  and  $H_2O_2$  concentrations. In addition, such heat-induced effects on antioxidant enzymes were also observed with SOD and POD isoforms, as Cu/ZnSOD maintained high stability under heat stress whereas the intensity of POD isoforms decreased with the duration of heat stress, especially at 47°C. Oxidative damage induced by heat stress was related to the changes in antioxidant enzyme activities and levels of antioxidants (Yin et al., 2008).

In plants, the AsA–GSH cycle plays a pivotal role in controlling ROS levels and cellular redox homeostasis. When AsA–GSH cycle enzymes were analyzed in the cytosol, mitochondria, and plastids of tobacco Bright Yellow-2 cultured cells subjected to two different heat shocks, it was observed that moderate heat shock (35°C) did not affect cell viability, whereas exposure of cells to 55°C led to heat shock-induced programmed cell death (PCD). In relation to AsA–GSH cycle, the three analyzed compartments appeared to have specific enzymatic profiles that were diversely altered by the heat shock. The cytosol contained the highest activity of all AsA–GSH cycle enzymes, in particular, the cytosolic APX was found to be the most versatile enzyme, the activity of which was enhanced after moderate heat shock and declined during PCD induction, whereas other APX isoenzymes were affected only in the cells undergoing PCD (Locato et al., 2009).

#### 5.4.4 CHILLING

Chilling stress is a key factor limiting the survival of plants and their geographical distribution. Susceptibility to chilling injury prevents the cultivation of many crops in regions where temperatures can drop much below the optimal growth temperatures (Ercoli et al., 2004). Chilling stress causes both preharvest and postharvest damage to plants, which results in enormous yield losses every year. Evidences suggest that chilling stress in plants leads to increased formation of ROS in both roots and leaves. Chilling conditions exacerbate the imbalance between light absorption and light use by inhibiting Calvin–Benson cycle activity (Logan et al., 2006). Enhanced photosynthetic electron flux to  $O_2$  and the over-reduction of respiratory electron transport chain resulting in ROS accumulation has been shown in cucumber leaves subjected to chilling (Hu et al., 2008). Oxidative stress has been suggested to be a significant factor in relation to chilling injury in plants. Chilling-associated oxidative damage that enhances the production of ROS slows down metabolism and

causes the peroxidation of membrane lipids and results in a significant increase in MDA content. The chilling of *Arabidopsis thaliana* (L.) Heynh. callus tissues to 4°C led to conditions of oxidative stress, as indicated by increased levels of the products of peroxidative damage to cell membranes (O’Kane et al., 1996). Evidence for chilling-induced oxidative stress has also been observed in maize seedlings (Prasad et al., 1994).

Responses to chilling-induced oxidative stress include alteration in the activities of enzymes of antioxidant defense system. In leaves of strawberry plantlets under low temperature treatment, the rates of  $O_2^{\cdot-}$  generation and the contents of  $H_2O_2$  and MDA increased and the activities of antioxidant enzymes SOD, CAT, POD, and APX gradually increased to a certain degree and decreased thereafter. Therefore, it is suggested that low temperature stress triggers increased production of ROS in plants, and the early accumulation of ROS might lead to increased activity of antioxidant defense system. If the duration of chilling stress is too long, the defense system may not remove overproduced ROS effectively, which may result in severe damage or even death (Yong et al., 2008). Chilling stress preferentially enhances the activities of the antioxidative enzymes SOD, APX, GR, and GPX, whereas the activity of CAT decreases in the leaves of cucumber seedlings (Lee and Lee, 2000). Nonenzymic antioxidants (AsA, GSH, and carotenoids) also play an important role in cold response. Under cold stress conditions, the content of low-molecular-weight antioxidants, especially that of reduced AsA, increases in barley seedlings. After the termination of stress, the contents of total AsA, GSH, and carotenoids get reduced to the level close to the initial one. Though the level of reduced AsA declines, it remains at the level higher than the initial. Therefore, reduced AsA has been suggested to be an important component in plant cell defense during low temperature treatment (Radyuk et al., 2009).

ROS producing and scavenging systems are found in various organelles. Radyuk et al. (2009) observed more active cytoplasmic SOD than its chloroplast isoforms in green barley (*Hordeum vulgare* L.) seedlings, suggesting that oxidative process initiation under low temperature stress occurred more actively in the cytosol compared to the chloroplasts. The response timing of different antioxidant enzyme isoforms to chilling was found to be different in leaves of cucumber seedlings. Out of five APX isoforms present in leaves, the intensities of APX-4 and -5 were enhanced by chilling stress, whereas that of APX-3 was significantly increased in the poststress periods after chilling stress. Expressions of Mn-SOD-2 and -4 were enhanced only after 48 h of the poststress period. All six GR isoforms showed increased intensity in stressed plants compared to the control and poststressed plants (Lee and Lee, 2000).

Cold treatment may initially cause injury, but during the recovery period, leaves coordinate and enhance the capacity of the antioxidative system, to diminish the potential of active oxygen species (Bafeel and Ibrahim, 2008). In lucerne leaves, after dark chilling treatment, a marked increase in the level of  $H_2O_2$  and MDA was observed (Bafeel and Ibrahim, 2008). After recovery period, the MDA content decreased significantly due to the increase of phenolic compounds, which suppressed lipid peroxidation. Also, the redox properties of  $\alpha$ -tocopherol play an important role in adsorbing and neutralizing free radicals and provide some forms of antioxidant protection. The activity of SOD increased sharply with the imposition of chilling stress, whereas CAT, APX, and GR activities slightly increased with the imposition of chilling. During the recovery period, activities of CAT, APX, and GR increased significantly, which could possibly restrict the recycling of active oxygen species associated with chilling stress (Bafeel and Ibrahim, 2008). Radyuk et al. (2009) concluded that APX and CAT play an important role in plant cell defense against low temperatures, whereas reduced GR and SOD activities are especially important during poststress period in barley seedlings.

Acclimation to prolonged chilling stress can be achieved by briefly pre-exposing the plants to low nonfreezing temperatures, a process called cold acclimation (CA). For a chilling-sensitive crop like maize, low temperature treatment during the early stages of development can be detrimental to subsequent crop establishment and productivity (Stewart et al., 1990; Greaves, 1996). Acclimation-induced CAT seems to play a major role, probably along with other antioxidant enzymes, in inducing chilling tolerance in pre-emergent maize seedlings (Prasad, 1997).

### 5.4.5 METAL TOXICITY

High concentrations of essential as well as nonessential metals in the soil environment, arising from mining and industrial activities, disposal of sewage sludge or soil acidification, and use of pesticides and fertilizers, continues to be a serious risk for plant health (Barcelo and Poschenrieder, 1990). Soil contamination with a variety of metals has become a global problem, leading to losses in agricultural yield (Salt et al., 1995). These metals are taken up from the soil by growing plants, and one of the consequences of the presence of the toxic metals within the plant tissues is the formation of free radical species, which can be initiated directly or indirectly by the metals and, consequently, lead to oxidative damage to different cell constituents (Gallego et al., 2002). Metal toxicity for living organisms involves oxidative and/or genotoxic mechanisms (Briat and Lebrun, 1999). Enhanced generation of ROS can overwhelm cells' intrinsic antioxidant defenses, and may result in a condition known as "oxidative stress." Recent studies indicate that transition metals act as catalysts in the oxidative reactions of biological macromolecules; therefore, the toxicities associated with these metals might be due to oxidative tissue damage. Both redox-active as well as redox-inactive metals cause an increase in the production of ROS such as  $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$ . Redox-active metals, such as iron, copper, and chromium, undergo redox cycling, whereas redox-inactive metals, such as lead, cadmium, mercury, and others deplete cell's major antioxidants, particularly thiol-containing antioxidants and enzymes (Gallego et al., 1996; Weckx and Clijsters, 1996; Yamamoto et al., 1997; Shah et al., 2001; Verma and Dubey, 2003; Sharma and Dubey, 2007; Maheshwari and Dubey, 2009). If metal-induced production of active oxygen species is not adequately counterbalanced by cellular antioxidants, the oxidative damage of lipids, proteins, and nucleic acids ensues (Halliwell and Gutteridge, 1989; Dat et al., 2000; Sharma and Dubey, 2007; Sandalio et al., 2009; Sharma and Dietz, 2009). A significant decline in protein thiol content is observed when rice seedlings are subjected to Al or Ni toxicity (Sharma and Dubey, 2007; Maheshwari and Dubey, 2009). In addition to the use of plant seedlings or adult plants, another interesting approach has been the use of *in vitro* cell cultures to study plant responses to abiotic stresses, more importantly metal toxicities (Gomes-Júnior et al., 2006). The induction of ROS production with heavy metals (cadmium and zinc) in *Nicotiana tabacum* L. cv. Bright Yellow 2 (TBY-2) cells in suspension cultures showed properties comparable to the elicitor-induced oxidative burst in other plant cells (Zrobek-Sokolnik et al., 2009). These heavy metals generated  $O_2^{\cdot-}$ ,  $H_2O_2$ . The effects of CAT, N,N-diethyldithiocarbamate (DDC), and SOD on the heavy metal-induced ROS production indicated that it occurs outside of the cells, and that at least part of the  $H_2O_2$  is produced by the dismutation of the  $O_2^{\cdot-}$ . Results suggested that the enzyme responsible for cadmium and zinc-induced ROS generation in tobacco cells contains a flavocytochrome (Zrobek-Sokolnik et al., 2009).

The increased activity of antioxidative enzymes in metal-stressed plants appears to serve as an important component of antioxidant defense mechanism of plants to combat metal-induced oxidative injury (Shah et al., 2001). Antioxidant system consisting of several nonenzymic and enzymic components is activated in the cells as a response to metal stress (Gallego et al., 1996; Shah et al., 2001; Verma and Dubey, 2003). Activation of antioxidant enzymes like POD, SOD, APX and enzymes of AsA-GSH cycle due to the toxicity of metals like Cd, Pb, Al, have been reported in plants by various groups of workers (Cakmak and Horst, 1991; Shah et al., 2001; Verma and Dubey, 2003; Sharma and Dubey, 2007). However, results suggest that the activation of antioxidant enzymes in response to oxidative stress induced by metals is not enough to confer tolerance to metal accumulation. Responses of heavy metal exposure to plants vary depending on plant species, tissues, stages of development, type of metal and its concentration. One of the key responses includes the triggering of a series of defense mechanisms that involve enzymatic and nonenzymatic components (Gratão et al., 2005). Among various metals like Cd, Pb, Al, and the metalloid As, different effects have been observed on plant metabolism, and although roots are regarded as the main sites of metal accumulation, the oxidative damage was found to be more detrimental in the leaves (Erdei et al., 2002; Jha and Dubey, 2004; Sharma and Dubey, 2007).

$\text{Cu}^{2+}$  ions are redox active and catalyze Fenton-type reactions producing  $\cdot\text{OH}$  (Elstner et al., 1988). Lipid peroxides also originate from the induction of the enzyme lipoxygenase in the presence of  $\text{Cu}^{2+}$  (Somashekaraiah et al., 1992). This enzyme is known to initiate lipid peroxidation. Iron has a pivotal and dual role in free radical chemistry in all organisms. Free Fe can participate in Fenton reactions and can catalyze the generation of  $\cdot\text{OH}$  and other toxic oxygen species. On the other hand, Fe is a constituent of the antioxidant enzymes CAT, APX, GPX, and Fe-SOD (Becana et al., 1998). When plants are exposed to a variety of adverse environmental conditions, including chilling, high light, drought, etc., oxidative stress occurs primarily due to the increase in free radical production mediated by catalytic Fe (Arora et al., 2002), and also due to decrease in antioxidative defense system (Arora et al., 2002).

Arsenic toxicity has been known for centuries, and has recently received increased attention because of its chronic and epidemic effects on human health (Abernathy et al., 1999). Plants normally take up arsenic predominantly in trivalent (AsIII) and pentavalent (AsV) forms, which are known to interfere with various metabolic pathways in cells, like, interaction with sulfhydryl groups, replacement of phosphate from ATP, and the excessive production of ROS. Singh et al. (2007) showed that As induces oxidative stress resulting from enhanced lipid peroxidation in mung bean (*Phaseolus aureus* Roxb.). The accumulation of MDA in seedlings increased significantly with increasing arsenic concentration (both AsIII and AsV). However, oxidative stress was more pronounced in As(III) treatment. The As treatments significantly increased the activities of antioxidant enzymes (SOD and GR) and the contents of antioxidant metabolites (GSH and AsA) in Indian mustard, the increase being dependent on exposure time. Increase in the activity of CAT was not significant. It was concluded that Indian mustard was able to detoxify the low As level through the induction of antioxidant defense mechanism (Khan et al., 2009). The upregulation of APX and POD has also been reported in As(III)/As(V) treatments. Both GSH and Cys imparted enhanced tolerance to seedlings against arsenic stress. Seedlings growth improved while the level of MDA declined significantly when GSH and Cys were supplemented to As(III)/As(V) treatments, suggesting GSH- and Cys-mediated protection against oxidative stress (Shri et al., 2009). While As (V) predominantly stimulates antioxidant enzyme activity, As (III) primarily causes enhanced levels of thiols (Srivastava et al., 2007).

Cadmium is a potent heavy metal pollutant of the environment. It is a heavy metal of widespread occurrence. Cadmium induces oxidative stress in several plant species and increases the activity of enzymes of antioxidant defense system (Shah et al., 2001; Metwally et al., 2003). Lipids that contain phosphate groups are essential components of membrane that surround the cell as well and other cellular structures, such as the chloroplast, mitochondria, and nucleus. Cd-induced oxidative damage involves the peroxidation of PUFA of membrane lipids due to ROS generated by Cd (Lin et al., 2007). Lipid peroxidation eventually increases membrane fluidity and membrane permeability. Elevated levels of lipid peroxides were observed in Cd-stressed rice seedlings (Shah et al., 2001). Under 500  $\mu\text{M}$  Cd treatment, about 1.4 to 1.6 times increase in MDA content was observed, indicating enhanced peroxidation of lipids due to Cd exposure (Shah et al., 2001). The increased production of ROS under Cd toxicity serves as a major source of DNA damage leading to strand breakage, removal of nucleotides, and a variety of modifications in the organic bases of nucleotides (Sarkar, 1995). A distinct pattern of DNA fragmentation, typical for PCD was observed in Cd-exposed tobacco cells (Fojtova and Kovarik, 2000). DNA damage was suggested to be an important mechanism of Cd phytotoxicity in *Vicia faba* plants (Lin et al., 2007). Cadmium treatment decreases chlorophyll and heme levels of germinating mung bean seedlings by the induction of lipoxygenase with the simultaneous inhibition of antioxidative enzymes, SOD and CAT (Somashekaraiah et al., 1992). Such inhibition results from the binding of the metal to the important sulfhydryl group of enzymes, which increases the phytotoxic action of metals (Van Assche and Clijsters, 1990). In pea plants, Cd toxicity induced carbonylation in proteins and the extent of carbonylation was greater in peroxisomes than in the whole plant (Romero-Puertas et al., 2002). Increased protein oxidation was observed in cucumber seedlings (*Cucumis sativus* L.) grown in increasing Cd levels. The importance of the

enzymatic and nonenzymatic antioxidant system in response to Cd toxicity has been shown by several authors. Enhancement in the activities of SOD, CAT, POD, and GR is observed in Cd-stressed rice seedlings (Shah et al., 2001). The activity of another antioxidative enzyme CAT increased in rice seedlings grown at moderately toxic Cd (100  $\mu$ M) level, whereas with highly toxic Cd (500  $\mu$ M) level, a marked inhibition in CAT activity was noted (Shah et al., 2001). Decline in CAT activity in plants growing under higher levels of Cd was suggested due to the inhibition of enzyme synthesis or a change in the assembly of enzyme subunits (Shah et al., 2001). Barley seedlings exposed to 25  $\mu$ M Cd showed increased activity of H<sub>2</sub>O<sub>2</sub>-metabolizing enzymes, CAT and APX (Metwally et al., 2003). Apparently, it is the oxidative stress induced by Cd that enhances the activities of stress-related enzymes by increasing the levels of free radicals and peroxides. Activity of APX was inhibited while the activities of CAT and SOD were increased in cucumber seedlings (*Cucumis sativus* L.) grown in increasing Cd levels. Simultaneously, AsA and nonprotein thiol groups were also found to increase (Gonçalves et al., 2007). A comparison of closely related plant species with different degrees of sensitivity to toxic metals has established a link between the degree of plant tolerance to metals and the level of antioxidants (Sharma and Dietz, 2009). Nouairi et al. (2009) showed that *Brassica juncea* plants possessed greater potential for Cd accumulation and tolerance than *Brassica napus* plants. *Brassica napus*, on Cd exposure, exhibited an increased level of lipid peroxidation, whereas in *Brassica juncea* treated plants, MDA content remained unchanged. In *Brassica napus*, with the exception of GPX, activity levels of some antioxidant enzymes involved in the detoxification of ROS, including SOD, CAT, GR, and APX, decreased drastically at high Cd concentrations. By contrast, in leaves of Cd-exposed *Brassica juncea* plants, there was either only little or no change in the activities of the antioxidative enzymes. Analysis of the profile of anionic isoenzymes of GPX revealed qualitative changes occurring during Cd exposure for both species. The mung bean genotypes (Pusa 9531, Pusa 9072, Pusa Vishal and PS-16) treated with Cd (0, 25, 50, and 100 mg/kg soil) showed a differential response to Cd concentrations; Pusa 9531 was identified as Cd tolerant, whereas PS 16 was Cd susceptible. The results revealed the presence of a strong antioxidant defense system (elevated activities of SOD, CAT, APX, and GR, and the increased amounts of AsA and GSH) in the Cd-tolerant genotype (Pusa 9531) for providing adequate protection against oxidative stress induced by Cd (Anjum et al., 2008).

Aluminum is a major constraint, reducing crop productivity in acid soils throughout the world (Kochian, 1995; Pereira et al., 2006). Although Al itself is not a transition metal and cannot catalyze redox reaction, the involvement of oxidative stress in Al toxicity has been suggested in many plant species. Even without an external supply of Fe, enhanced peroxidation of lipids is observed due to Al in pea (*Pisum sativum*) roots (Yamamoto et al., 2001). Al ions have a strong affinity for biomembranes and cause the rigidification of membranes (Deleers et al., 1986), which seems to facilitate the radical chain reaction, enhancing the peroxidation of lipids in phospholipid liposomes (Oteiza, 1994), soybean (*Glycine max*) roots (Cakmak and Horst, 1991), and cultured tobacco (*Nicotiana tabacum*) cells (Ono et al., 1995; Yamamoto et al., 1997). The Al-induced peroxidation of lipids leads to loss of plasma membrane integrity and eventually cell death in cultured tobacco cells (Yamaguchi et al., 1999). Boscolo et al. (2003) observed a higher degree of protein oxidation in maize roots compared to lipid peroxidation, in Al-treated plants and suggested that in maize roots proteins are the primary target of damage due to ROS under Al toxicity. Sharma and Dubey (2007) also observed the increased modification of proteins in rice seedlings subjected to Al toxicity. Al treatment led to DNA fragmentation in rice seedlings (Meriga et al., 2004). Signaling pathways involving H<sub>2</sub>O<sub>2</sub> cause PCD in heavy metal-stressed plants. Cells treated with 100  $\mu$ M AlCl<sub>3</sub> showed typical features of PCD such as nuclear and cytoplasmic condensation in tomato (*Lycopersicon esculentum* Mill.) suspension cells. Cell death was suppressed by the application of antioxidants and by inhibitors of phospholipase C (PLC), phospholipase D (PLD), and ethylene signaling pathways. The results suggest that low concentrations of heavy metal ions stimulate both PLC and PLD signaling pathways, leading to the production of ROS and subsequent cell death executed by caspase-like proteases (Yakimova et al., 2007).

As Al induces the expression of diverse genes in plant species like wheat, maize, sugarcane, tobacco, *Arabidopsis* and many of these genes encode antioxidant enzymes such as glutathione S-transferase, peroxidase, SOD (Ezaki et al., 2000; Simonovicova et al., 2004). A strong correlation has been suggested between Al toxicity and oxidative stress in plants (Richards et al., 1998; Boscolo et al., 2003; Watt, 2004). Ezaki et al. (2000) confirmed this hypothesis when they showed that over-expression of some Al-induced genes in transgenic *Arabidopsis* plants conferred oxidative stress resistance. Meriga et al. (2004) found a close inverse relationship between decreased root growth and increased Al accumulation, lipid peroxidation, SOD and POD activities, and DNA damage in rice plants. Higher activities of SOD and POD in Al-stressed rice seedlings suggested that these enzymes could serve as efficient free radical scavengers to minimize the adverse effects of lipid peroxidation and could contribute to the maintenance of membrane structure and the integrity of rice plants under Al toxicity. According to Sharma and Dubey (2007), Al toxicity is associated with the induction of oxidative stress in rice plants, and among antioxidative enzymes, SOD, guaiacol POX, and cytosolic APX appear to serve as important components of antioxidative defense mechanism under Al toxicity. Polyacrylamide gel electrophoresis (PAGE) confirmed the increased activity as well as the appearance of new isoenzymes of APX in Al-stressed seedlings. Immunoblot analysis revealed that changes in the activities of APX are due to changes in the amounts of enzyme protein (Sharma and Dubey, 2007).

Lead is one of the major heavy metals of antiquity and has gained considerable importance as a potent environmental pollutant (Sharma and Dubey, 2005). One of the phytotoxic effects of Pb appears to be the induction of oxidative stress in growing plant parts due to the enhanced production of ROS resulting in unbalanced cellular redox status. Pb is not a oxido-reducing metal like iron, therefore, the oxidative stress-induced by Pb in plants appears to be an indirect effect of Pb toxicity leading to the production of ROS, enhancing the pro-oxidant status of the cell by reducing the pool of reduced GSH, activating calcium-dependent systems, and affecting iron-mediated processes (Pinto et al., 2003). Though the ROS-generating processes are slow under normal conditions, Pb accelerates them (Verma and Dubey, 2003). Such production depends on the intensity of stress, repeated stress periods, species and age of plants (Asada, 1994; Verma and Dubey, 2003). Pea plants treated with Pb showed the increased level of the ROS  $O_2^{\cdot-}$  and  $H_2O_2$  in the roots and the increase was proportional to metal concentration (Malecka et al., 2009). Lipid peroxidation, which is regarded as an indicator of oxidative damage, involves the oxidative degradation of PUFA residues of membranes (Girotti, 1990). Pb ions induce lipid peroxidation, decrease the level of saturated fatty acids, and increase the content of unsaturated fatty acids of membrane in several plant species (Halliwell and Gutteridge, 1999; Verma and Dubey, 2003; Reddy et al., 2005). MDA level was reported to increase concomitantly with the increasing level of  $H_2O_2$  produced in pea plants treated with Pb (Malecka et al., 2009). When rice (*Oryza sativa*) seedlings were raised in sand cultures under 500 and 1000  $\mu M$   $Pb(NO_3)_2$  in the medium, during a growth period of 5–20 days, about 21%–177% increase in the level of lipid peroxides was observed, indicating that Pb induces oxidative stress in rice plants (Verma and Dubey, 2003).

The importance of antioxidant defense mechanism to tolerate oxidative stress produced by Pb has been shown by various research groups. Activities of the antioxidative enzymes SODs, CAT, and APX and the levels of low-molecular antioxidants, particularly GSH, homogluthathione (h-GSH), and cysteine were reported to increase concomitantly with increasing Pb concentration and the duration of treatment (Malecka et al., 2009). The redox state (GSH/GSSG) of root cells dropped proportionally to stress intensity (Malecka et al., 2009). Pb-treated rice seedlings showed increase in the activities of the enzymes SOD, GPX, APX, and GR compared to untreated controls (Verma and Dubey, 2003). A highly toxic concentration of Pb (1000  $\mu M$ ) treatment led to decrease in the intensity of two pre-existing CAT isoforms in shoots of rice seedlings. It is shown that Pb induces oxidative stress in growing rice plants and that SOD, PODs, and GR could serve as important components of antioxidative defense mechanism against Pb-induced oxidative injury in rice plants (Verma and Dubey, 2003). Chloroplasts isolated from spinach seedlings treated with  $PbCl_2$  showed increase in both ROS and MDA content, and reduction in photosynthesis and activities of the antioxidant defense system (SOD, CAT, APX, GPX, and GSH content), indicating that spinach

chloroplasts underwent a stress condition due to an oxidative attack. The results imply that spinach chloroplasts were not able to tolerate the oxidative stress induced by  $\text{Pb}^{2+}$  possibly due to lack of an effective antioxidant defense mechanism (Xiao et al., 2008a).

Nickel is an essential nutrient for plants. However, the amount of Ni required for normal growth of plants is very low. Nickel toxicity has been associated with oxidative stress in plants. Nickel-treated rice seedlings showed increased rates of  $\text{O}_2^{\cdot-}$  production, elevated levels of  $\text{H}_2\text{O}_2$ , and thiobarbituric acid reactive substances (TBARS), demonstrating enhanced lipid peroxidation, and a decline in protein thiol levels indicative of increased protein oxidation compared to controls (Maheshwari and Dubey, 2009). Gajewska and Skłodowska (2007) observed that despite prolonged increases in  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  levels, oxidative damage measured in terms of lipid peroxidation, did not occur in the leaves of Ni-treated wheat plants.

With progressively higher Ni concentrations, nonprotein thiol and AsA levels increased, whereas the level of low-molecular-weight thiols (such as GSH and hydroxyl-methyl GSH), the ratio of these thiols to their corresponding disulfides, and the ratio of AsA to DHA declined in the rice seedlings (Maheshwari and Dubey, 2009). The activities of all isoforms of SOD (Cu-Zn SOD, Mn SOD, and Fe SOD), GPX, and APX were reported to increase in Ni-treated rice seedlings, while no clear alteration in CAT activity could be observed (Maheshwari and Dubey, 2009). Excess supply of Ni was shown to inhibit the CAT activity and induce POD, APX, and SOD activities in maize. Localization of isoforms of these enzymes on native gels also revealed increases in the intensities of pre-existing bands. Enhanced activities of peroxidase, APX, and SOD, however, did not appear to be sufficient to ameliorate the effects of excessively generated ROS due to excess supply of Ni (Kumar et al., 2007). Activity of the AsA-GSH cycle enzymes MDHAR, DHAR, and GR significantly increased in Ni-treated seedlings (Maheshwari and Dubey, 2009). Exposure of Ni to *Nicotiana tabacum* cv. BY-2 cell suspensions led to alterations in isoenzymes of SOD, CAT, and GR. Activity staining analysis revealed that CAT activity plays a major role in the early response to Ni-induced oxidative stress, particularly, when the Ni concentration used is low, while a specific GR isoenzyme appears to respond to the Ni-induced oxidative stress when a much higher Ni concentration is used to induce the stress for the same period of treatment (Pompeu et al., 2008). These results illustrate the importance and advantages of determining individual isoenzyme activities. The differential responses of SOD, CAT, and GR isoenzymes are associated with specific physiological phenomena of the cells due to the specific organellar localization of the isoenzymes (Pompeu et al., 2008).

When wheat seedlings were treated with Ni, a significant increase in  $\text{H}_2\text{O}_2$  concentration was observed in both roots and shoots (Gajewska and Skłodowska, 2008). The constitutive activities of antioxidative enzymes, other than CAT, were much higher in the roots than in the shoots, and Ni application caused several-fold increased activities of APX, POD, and GST in the shoots, whereas in the roots, the activities were not significantly altered. The differential antioxidative responses of the shoots and roots of wheat seedlings to Ni stress might be ascribed to diverse constitutive levels of antioxidant enzyme activities in both the organs (Gajewska and Skłodowska, 2008). When  $\text{H}_2\text{O}_2$  levels were compared in hairy roots of hyperaccumulator species, *Alyssum bertolonii* and the nonhyperaccumulator, *Nicotiana tabacum*, it was found that  $\text{H}_2\text{O}_2$  levels rose significantly with Ni treatment in both species, by factors of 3.6 and 8.6, respectively. Compared with *N. tabacum*, oxidative damage was suggested to be minimized in *A. bertolonii* roots by the high endogenous activities of CAT and, to a lesser extent, SOD (Boominathan and Doran, 2002). Using a transgenic approach, it has been confirmed that antioxidative defense system plays an important role in combating metal-induced oxidative stress in plants (Duan et al., 2006; Guan et al., 2009).

#### 5.4.6 ANAEROBIOSIS

Anaerobic stress is thought to be a major stressful factor in the growth of crop plants in waterlogged soils, as oxygen diffuses 10,000 times more slowly in water than in air (Armstrong, 1979). Oxygen deficiency causes stomatal closure (Crawford, 1978), which reduces  $\text{CO}_2$  availability in the leaves

and inhibits carbon fixation. Thus, excessive excitation energy in chloroplasts could increase the generation of ROS and induce oxidative stress (Gossett et al., 1994). An excessive accumulation of  $O_2^{\cdot-}$  and  $H_2O_2$  is observed in plants subjected to anaerobic stress (Yan et al., 1996; Kumutha et al., 2009; Sairam et al., 2009). Roots suffer from the periodic or prolonged deprivation of oxygen, which leads to diminished respiration at the level of electron transport. The lack of a suitable electron acceptor leads to saturated redox chains, accumulation of NAD(P)H, and a decline in the generation of ATP. Flooding stress also contributes to the accumulation of acetaldehyde and other compounds derived from the anaerobic metabolism, which could be susceptible to degradation, yielding  $H_2O_2$  as an end product (Blokhina et al., 2003). The presence of  $H_2O_2$  in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions in yellow flag iris (*Iris pseudacorus*) and rice, and anoxia-intolerant wheat and garden iris (*Iris germanica*) (Blokhina et al., 2001). In the anoxia-tolerant species, the response was delayed in time, and in highly tolerant *I. pseudacorus*, plasma membrane-associated  $H_2O_2$  was detected only after 45 days of oxygen deprivation.

Waterlogging-induced production of  $H_2O_2$  is also involved in the signaling and induction of various defense-related genes, leading to the synthesis of proteins/enzymes imparting hypoxia tolerance (Sairam et al., 2009). Excessive production of ROS can oxidize biological molecules, such as DNA, protein, and lipids, causing the malfunction of these molecules (Richter and Schweizer, 1997). Oxidative stress is an integral part of oxygen deprivation stress. Enhanced lipid peroxidation products have been observed in plants under low oxygen condition (Yan et al., 1996; Chirkova et al., 1998; Blokhina et al., 1999). The increased level of TBARS was observed at 4 and 6 days of waterlogging in pigeon pea plants over control plants, probably due to the activation of diphenyleneiodonium-sensitive NADPH oxidase (Kumutha et al., 2009). The injury of biological lipids by ROS, as indicated by MDA content, was clearly detected in the waterlogged barley plants, but with distinct difference between the Xiumai 3 (tolerant) and Gerdner (sensitive) genotypes, implying the possible difference in the capacity of their defense systems against ROS (Zhang et al., 2007). Thus, the sensitivity of plants to environment stress, including waterlogging, has been considered to be associated with antioxidative ability (Crawford, 1978; Foyer et al., 1994). Jamei et al. (2009) showed that the excessive accumulation of  $H_2O_2$ - and  $O_2^{\cdot-}$ -induced membrane damage and lipid peroxidation in leaves of *Zea mays* subjected to hypoxia was the result of the reduced activity of SOD.

When the activities of antioxidant enzymes were examined for the waterlogging tolerance of pigeon pea (*Cajanus cajan* L. Halls) genotypes ICP 301 (tolerant) and Pusa 207 (sensitive), it was observed that the activities of enzymes SOD, APX, GR, and CAT increased under waterlogging in both the cultivars and that, comparatively, greater antioxidant enzyme activities resulting in less oxidative stress were observed in the tolerant genotype ICP 301 compared to the sensitive genotype Pusa 207, suggesting that the greater activities of antioxidative enzymes represent one of the factors determining the higher tolerance of pigeon pea plants to flooding (Kumutha et al., 2009). Lin et al. (2008) observed that increased APX activity, increased levels of total GSH, oxidized, and total AsA at different days of flooding, confer flooding tolerance to the sweet potato plants (*Ipomoea batatas* L. Lam. "Tainung 57"). When eggplant genotypes EG117 and EG203, and tomato genotypes, L4422 and TNVEG6, were subjected to seven flooding treatments, the activity of APX in roots significantly increased during the period of continuous waterlogging (Lin et al., 2004). Slight increases in total AsA, reduced AsA, GSH, and total GSH contents in the roots were also observed throughout the entire waterlogging period. However, the activities of CAT, SOD, and GR, and the contents of AsA, GSSG, and  $\alpha$ -tocopherol in the roots were unaffected by waterlogging (Lin et al., 2004). Genotypes responded differently to oxidative injury according to the activity of their different antioxidative components. Following waterlogging treatments, APX activity in the eggplants was generally higher than in tomatoes. Limiting or less efficient APX in the tomatoes, led to the accumulation of  $H_2O_2$ . It was concluded that increased APX activity could provide increased tolerance to waterlogging in the plants (Lin et al., 2004).



Rice seedlings raised under water for 6 days showed reduced activities of SOD, APX, MDHAR, DHAR, GR, and CAT compared to seedlings raised in air for the same period of time (Ushimaru et al., 1992). AsA and GSH were present in submerged seedlings at nearly the same levels as those found in aerobically grown controls. When submerged, seedlings were exposed to air; the activities of the six antioxidative enzymes exceeded the levels in aerobically grown controls, during 24 h of adaptation to air. The level of AsA increased slightly, but the level of GSH showed a rapid increase, reaching seven times that in aerobically grown controls within 12 h of adaptation to air. Therefore, in this case, the development of antioxidative defense system was proposed to consist of two steps, namely, a rapid increase in the level of GSH and a subsequent slow increase in the activities of antioxidant enzymes (Ushimaru et al., 1992). The antioxidant systems of root and leaf cells behave differentially to changes in oxygen concentration. In leaves, low-mass antioxidants play the major role in ROS detoxification, while in roots, increased alternative oxidase capacity supports the antioxidant systems, possibly by preventing ROS formation (Skutnik and Rychter, 2009).

When the responses of antioxidant enzymes to the different depths of waterlogging in creeping bentgrass (*Agrostis stolonifera* L.) roots were examined, it was observed that SOD and APX were mainly involved in waterlogging-induced antioxidant responses, and that partial waterlogging could also significantly affect root antioxidant activities (Lin et al., 2004). The ability to maintain a balance between the formation and detoxification of ROS appears likely to contribute to the increased survival potential and the tolerance of the roots against oxidative stress (Lin et al., 2004).

#### 5.4.7 GASEOUS POLLUTANTS

The problem related to gaseous pollutants has increased during the recent years. Ozone, the most abundant air pollutant is detrimental to plants, because of its strong oxidizing potential. Ozone concentration and exposure time determine the chronic or acute toxicity and, consequently, the severity of injury at biochemical and physiological level. Ozone induces the formation of ROS in plants. Tropospheric ozone damages crop plants and forests by entering leaf mesophyll tissues through the stomata and rapidly generating ROS- $O_2^-$ ,  $\cdot OH$ ,  $H_2O_2$  at the cell perimeter (Pellinen et al., 1999; Pasqualini et al., 2002). Ozone that enters plants through the stomata is rapidly broken down into various ROS at the cell wall interface. Recently, however, it has been shown that ozone induces active ROS production. The ozone-induced accumulation of  $H_2O_2$  is observed initially on the plasma membrane and cell wall in birch (*Betula pendula*) (Pellinen et al., 1999). Experiments with inhibitors of possible sources for  $H_2O_2$  production in the cell wall suggested that both NADPH-dependent superoxide synthase and the cell wall POD are involved in such  $H_2O_2$  production. The  $H_2O_2$  production continued in the cytoplasm, mitochondria, and peroxisomes when the ozone exposure was over, but not in chloroplasts. The timing of mitochondrial  $H_2O_2$  accumulation coincided with the first symptoms of visible damage and, at the same time, the mitochondria showed disintegration of the matrix. These responses may not be directly connected with defense against oxidative stress, but may rather indicate changes in oxidative balance within the cells that affect mitochondrial metabolism and the homeostasis of the whole cell, possibly leading to the induction of programmed cell death (Pellinen et al., 1999). In sensitive plant species, even a few hours of exposure to this potent oxidant leads to severe oxidative stress that manifests the symptoms of visible cell death. Two mechanisms have been suggested for the injury brought about by ozone or ozone-induced ROS production: (1) modification of proteins (Rao et al., 1995), lipids (Calatayud and Barreno, 2001), and nucleic acids (Rousseaux et al., 1999), and (2) activation of programmed cell death pathway, similar to the pathogen-induced hypersensitive response (Overmyer et al., 2005). It has been postulated that ozone or ozone-induced ROS may locally cause necrotic cell death that, in turn, can trigger the PCD pathway in surrounding tissues. An acute exposure to 150 ppb ozone decreased stomatal conductance to 60%–70% of its initial value within 9–12 min in *Arabidopsis* plants (Kollist et al., 2007). The transient decrease was absent in the abscisic acid-insensitive mutant *abi2* defective in a class 2C protein phosphatase. This provides an *in vivo* confirmation that the early transient decrease

in stomatal conductance is not a result of physical damage by the ROS formed from ozone breakdown but reflects the biological action of ROS, transduced through a signaling cascade (Kollist et al., 2007). Highly significant interactions between ozone damage and levels of ROS, AsA, GSH, and lipid peroxidation were observed in *Medicago truncatula* accessions from various geographical regions. There were significant differences among the accessions for these traits before and after the end of ozone fumigation, suggesting that multiple physiological and biochemical mechanisms may govern ozone tolerance or sensitivity (Puckette et al., 2007). Acute ozone treatment (300 nL L<sup>-1</sup> for 6 h) led to a ROS burst in sensitive genotype Jemalong, 6 h postfumigation, whereas in resistant genotype JE154, increase in ROS levels was much reduced. Unique and shared transcriptional responses in an ozone-resistant and sensitive accession exemplify the complexity of oxidative signaling in plants (Puckette et al., 2007). It has been speculated that plants sensitive to acute ozone are impaired in the perception of the initial signals generated by the action of this oxidant. This, in turn, leads to a delayed transcriptional response in the ozone-sensitive plants. In resistant plants, the rapid and sustained activation of several signaling pathways enables the deployment of multiple mechanisms for minimizing the toxicity effect of this reactive molecule (Puckette et al., 2008).

In living organisms, ROS, directly or indirectly derived from ozone exposure, are scavenged by enzymatic and nonenzymatic antioxidant defensive mechanisms, overall deputed to preserve cell structures and macromolecules from the oxidative damage. These defenses are essentially those also involved in detoxifying the ROS inevitably produced by the metabolism of organisms living in oxygenic atmosphere (Iriti and Faoro, 2008). Studies on enzymatic and nonenzymatic antioxidants in two species belonging to a natural ecosystem, *Trifolium repens* L. (cv. Sonja) and *Trifolium pratense* L. (cv. Milvus) in response to an acute ozone dose, revealed that *T. repens* had higher constitutive levels of the defense molecules compared with the other species. Moreover, it proved to be more prone to activate the antioxidant systems in response to ozone, showing a lower degree of sensitivity to the pollutants (Scebba et al., 2003). Rao et al. (1996) observed that *A. thaliana* genotype Landsberg erecta was capable of metabolizing ozone-induced activated oxygen by invoking the enzymes of SOD/AsA–GSH cycle. Ozone exposure not only enhanced SOD, POD, GR, and APX activity but also modified the substrate affinity of both GR and APX (Rao et al., 1996). Ozone impacts on the yield and quality of crops is influenced by changing climatic conditions, increasing atmospheric CO<sub>2</sub>, and altered emission patterns (Fuhrer, 2009). Enhanced oxidative damage to proteins was observed in wheat plants (*Triticum aestivum* L. cv. Roblin) exposed to ozone under ambient CO<sub>2</sub> but not in plants exposed to ozone under high CO<sub>2</sub> (Rao et al., 1995). Ozone exposure initially enhanced the specific activities of SOD, POD, GR, and APX irrespective of growth in ambient or high CO<sub>2</sub>. However, the specific activities decreased in plants with prolonged exposure to ozone under ambient CO<sub>2</sub> but not in plants exposed to ozone under high CO<sub>2</sub>. Preferential changes in the isoform composition of SOD, POD, and APX was observed in plants grown under a combination of high CO<sub>2</sub> and ozone. A new isoform of GR was observed in plants grown under high CO<sub>2</sub> and ozone, which could be the reason for higher resistance of these plants to ozone-induced damage compared to plants exposed to ozone under ambient CO<sub>2</sub> (Rao et al., 1995). To study the relation between ozone sensitivity and leaf concentrations of antioxidants (AsA, total phenolics, and total antioxidant capacity), Severino et al. (2007) exposed ozone-sensitive (NC-S clone) and resistant plants (NC-R clone) of *Trifolium repens* and *Centaurea jacea* to moderate ozone concentrations in ambient air. NC-R clone showed the highest concentrations of antioxidants with 50%–70% more AsA than NC-S. NC-R had about five times more AsA in the young leaves and nine times more in the older leaves than *Centaurea*. In a fumigation experiment with acute ozone stress (100 nL L<sup>-1</sup>), the antioxidant levels changed profoundly. The ozone-injured leaves of NC-S had 6–8 times more total phenolics than uninjured leaves. Generally, older leaves had lower antioxidant concentrations and were more prone to ozone injury than younger leaves. Low AsA concentrations were closely related to the appearance of visible ozone injury than the other antioxidant parameters.

Sulfur dioxide (SO<sub>2</sub>) is a gaseous pollutant widely diffused in the world today. SO<sub>2</sub> penetrates in the leaves through the stomata. It dissolves in the aqueous medium surrounding the plant cells,

generating the toxic molecular species sulfite and bisulfite. The detoxification reaction of sulfite to sulfate, which takes place by reactions initiated by light, is mediated by the photosynthetic electron transport chain (Asada, 1980) and it leads to the formation of  $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$  (Asada, 1980). These highly oxidant molecular species, together with the toxic sulfite, can damage the lipids and proteins of cell membranes. The damage of leaves by  $SO_2$  in the presence of light has been attributed to the oxidative chain reaction of sulfite initiated by  $O_2^{\cdot-}$  produced as a result of the Mehler reaction in chloroplasts (Asada and Takahashi, 1987). Plants of two wheat (*Triticum aestivum* L.) cvs. namely "Mec" and "Chiarano," each with a different sensitivity to  $SO_2$ , when exposed to  $SO_2$ , revealed that different sensitivities to  $SO_2$  of the two cultivars were due to differential ability to maintain the elevated levels of AsA rather than increasing detoxifying enzyme activities (Ranieri et al., 1997). A significant increase in AsA, GSH, and their redox state was observed in plants exposed to high  $CO_2$  and  $SO_2$ , compared to that of plants exposed to solely  $SO_2$ . The absence of the negative effects of  $SO_2$  in the presence of high  $CO_2$  has been correlated to a high redox state of AsA and GSH (Rao and Dekok, 1994).

#### 5.4.8 UV-B RADIATIONS

The most damaging part of the UV spectrum reaching the earth's atmosphere is UV-B (290–320 nm). Increase in solar UV-B radiation reaching the earth's surface due to the depletion of the stratospheric ozone layer has become a major global concern (Blumthaler and Ambach, 1990; Xiong and Day, 2001). UV-B impairs several biochemical and physiological processes, including gene expression (Greenberg et al., 1989; Booij-James et al., 2000). UV-B radiation has a negative impact on plant cells, including enhanced generation of ROS, reduced photosynthesis, decreased protein synthesis, and impaired chloroplast functions (Strid et al., 1994; Booij-James et al., 2000; Han et al., 2009). UV-B causes oxidative stress through the excessive production of ROS  $O_2^{\cdot-}$ ,  $\cdot OH$ ,  $H_2O_2$  (Strid et al., 1994; Han et al., 2009), which, in turn, causes enhanced lipid and protein oxidation (Landry et al., 1995; Mittler, 2002; Babu et al., 2003; Frohnmeyer and Staiger, 2003; Yannarelli et al., 2006; Lu et al., 2009). The mechanisms of ROS generation by UV are not well understood (Green and Fluhr, 1995; Hideg et al., 2002). Rao et al. (1996) demonstrated that UV-B radiation enhances the activated oxygen species by increasing membrane-localized NADPH-oxidase activity and decreasing CAT activities (Rao et al., 1996). Plants must adapt to the deleterious effects of UV-B radiation because they are dependent on sunlight for photosynthesis and, therefore, cannot avoid exposure to UV-B radiation. Besides, antioxidative enzymatic scavengers for ROS such as SOD, POD, CAT, and APX, and nonenzymatic antioxidants like AsA and GSH, plants possess substances such as UV-B absorbing compounds and carotenoids to keep the balance between the production and removal of ROS (Costa et al., 2002; Han et al., 2009). In *Picea asperata* seedlings, enhanced UV-B (30%) induced the overproduction of ROS, leading to oxidative stress. UV-B increased the efficiency of antioxidant defense system consisting of UV-B absorbing compounds, carotenoids, and antioxidant enzymes SOD, APX, CAT, and POD (Han et al., 2009). UV-B exposure preferentially enhanced GPX, APX, and POD specific to coniferyl alcohol and modified the substrate affinity of APX in *A. thaliana*. New isoforms of PODs and APX were synthesized in flavonoid-deficient mutant transparent testa (*tt5*) of *A. thaliana* (Rao et al., 1996). Leaves from bitter melon (*Momordica charantia* L.) subjected to UV-B at three different stages of plant growth: preflowering, flowering, and postflowering revealed that the activities of SOD, CAT, POD, polyphenol oxidase, GR, and the concentrations of AsA,  $H_2O_2$ , and TBARS were elevated under UV-B exposure at all growth stages with the exception of  $H_2O_2$  concentration at the postflowering stage. It is suggested that *M. charantia* exhibits a protection mechanism against oxidative damage by maintaining a highly induced antioxidant system under UV-B stresses (Agarwal and Shaheen, 2007). Numerous studies have shown that large differences in UV-B sensitivity exist among plant species and even between cvs. of the same species (Teramura and Murali, 1986; Barnes et al., 1993). Santos et al. (1999) observed the influence of UV-B radiation on the activity of SOD and the number and amount of isoforms in the leaves of

C<sub>3</sub> plants, potato and wheat, and C<sub>4</sub> plants, maize and sorghum. The total specific activity of SOD increased significantly in wheat, maize, and potato, whereas a decline was induced in sorghum. Native gels revealed that UV-B caused preferential changes of the SOD isoforms in all plants. The rise in SOD activity in maize, potato, and wheat was correlated with the UV-B tolerance of these crops, and the sensitivity of sorghum to UV-B was associated with the decrease in SOD activity.

Gao and Zhang (2008) studied the response of an AsA-deficient *Arabidopsis thaliana* mutant *vtc1* to short-term increased UV-B exposure. After UV-B supplementation, *vtc1* mutants showed increase in H<sub>2</sub>O<sub>2</sub> content and the production of TBARS and a decrease in chlorophyll content and chlorophyll fluorescence parameters. The reduced ratio of GSH/total GSH and the increased ratio of DHA/total AsA were observed in the *vtc1* mutants, compared to the wild-type plants. In addition, the enzymes responsible for ROS scavenging, such as SOD, CAT, and APX and enzymes responsible for the regeneration of AsA and GSH (including MDHAR, DHAR, and GR) had insufficient activity in the *vtc1* mutants, compared to the wild-type plants. These results suggest that the AsA-deficient mutant *vtc1* is more sensitive to supplementary UV-B treatment than wild-type plants and AsA can be considered as an important antioxidant for UV-B radiation (Gao and Zhang, 2008).

## 5.5 PRODUCTION OF ABIOTIC STRESS-TOLERANT TRANSGENIC CROP PLANTS USING COMPONENTS OF ANTIOXIDATIVE DEFENSE SYSTEM

Oxidative stress is a key factor involved in the expression of abiotic stress-induced damages in crop plants. Maintaining redox balance is one of the crucial requirements for cells to endure abiotic stresses. Tolerance to several abiotic stresses has been correlated well with the enhanced level of components of antioxidant defense mechanism. Transgenic plants offer novel possibilities to achieve complete understanding of the roles of different enzymes and biomolecules involved in protection against the stresses of many types. Genetic engineering approaches have gained considerable grounds in terms of improving the traits of many crops within the shortest possible time period. Transgenic plants overexpressing enzymatic and nonenzymatic components of antioxidant defense system, singly or in combination, can be successfully used as attractive targets to produce abiotic stress-tolerant plants using biotechnological approaches.

The enhancement of the capacity of antioxidative defense system using gene transfer technology has shown to enhance the tolerance of crop plants to abiotic stresses, with improved performance and productivity under these stresses. Table 5.2 provides an overview of abiotic stress-tolerant plants produced by overexpressing the different enzymatic components of antioxidative defense mechanism. SOD serves as the first enzyme in the chain of enzymatic components of antioxidative defense system. Increased tolerance to some of the environmental stresses has been achieved by overexpressing SOD in target plants (Matters and Scandalios, 1986; Slooten et al., 1995a). Tolerance to drought (Badawi et al., 2004; Wang et al., 2005a), salinity (Liu et al., 2003; Prashanth et al., 2008; Zhang et al., 2008), low-temperature stress (Gupta et al., 1993; McKersie et al., 1993; Lee et al., 2009), and ozone (Van Camp et al., 1994) has been achieved by overexpressing SOD. The overexpression of wheat *TaSOD1.1* and *TaSOD1.2* genes increased SOD activities and decreased MDA content, resulting in less overoxidation of the cellular membrane system and the enhancement of physiological functions, with improved low-temperature stress tolerance and NaCl tolerance in transgenic tobacco plants (Zhang et al., 2008, 2009). Under salt stress, the upregulation of exotic target genes was also observed in transgenic plants at the transcriptional level, which could further enhance the salt-tolerant capacity of these plants (Zhang et al., 2008). Halophytic plants like mangroves have been reported to have a high level of SOD activity, which plays a major role in defending the mangrove species against severe abiotic stresses. *Sod1*, a cDNA encoding a cytosolic Cu/Zn-SOD from the mangrove plant *Avicennia marina* was expressed in rice. The transgenic plants withstood salinity stress of 150 mM of NaCl for a period of 8 days while the untransformed control plants wilted at the end of the stress treatment in hydroponics. The transgenic plants also revealed better tolerance to drought stress in comparison to untransformed control plants (Prashanth et al., 2008).

**TABLE 5.2**  
**Abiotic Stress-Tolerant Transgenic Plants Produced by Overexpressing**  
**Enzymes of Antioxidant Defense System**

| Antioxidant Enzyme  | Abiotic Stress        | Plants                         | References                  |
|---------------------|-----------------------|--------------------------------|-----------------------------|
| SOD                 | Salinity              | <i>Nicotiana tabacum</i>       | Zhang et al. (2008)         |
| Cu-Zn-SOD           | Water deficit         | <i>Nicotiana tabacum</i>       | Badawai et al. (2004)       |
|                     | Salinity              | <i>Oryza sativa</i>            | Prashanth et al. (2008)     |
|                     | Chilling              | <i>Oryza sativa</i>            | Lee et al. (2009)           |
|                     | Chilling              | <i>Zea mays</i>                | Van Breusegem et al. (1999) |
| Fe-SOD              | Freezing              | <i>Medicago sativa</i>         | McKersie et al. (2000)      |
|                     | Ozone                 | <i>Nicotiana tabacum</i>       | Van Camp et al. (1994)      |
| Mn-SOD              | Salinity              | <i>Arabidopsis thaliana</i>    | Wang et al. (2004)          |
| CAT                 | Drought and chilling  | <i>Lycopersicon esculentum</i> | Mohamed et al. (2003)       |
|                     | Salinity              | <i>Oryza sativa</i>            | Nagamiya et al. (2007)      |
|                     | Salinity              | <i>Nicotiana tabacum</i>       | Moriwaki et al. (2008)      |
|                     | Cadmium               | <i>Nicotiana tabacum</i>       | Guan et al. (2009)          |
| APX                 | Salinity and chilling | <i>Lycopersicon esculentum</i> | Wang et al. (2005b)         |
|                     | Salinity              | <i>Arabidopsis thaliana</i>    | Lu et al. (2007)            |
| Cytosolic-APX       | Salinity              | <i>Nicotiana tabacum</i>       | Sun et al. (2009)           |
| Thylakoid-bound APX | Cadmium               | <i>Oryza sativa</i>            | Duan et al. (2006)          |
| Peroxisomal APX     | Salinity              | <i>Arabidopsis thaliana</i>    | Xu et al. (2008a)           |
|                     | Zinc                  | <i>Arabidopsis thaliana</i>    | Xu et al. (2008b)           |
|                     | Drought               | <i>Nicotiana tabacum</i>       | Eltayeb et al. (2007)       |
| MDHAR               | Salinity              |                                |                             |
|                     | Ozone                 |                                |                             |
|                     | Salinity and chilling | <i>Nicotiana tabacum</i>       | Kwon et al. (2003)          |
| DHAR                | Salinity              | <i>Arabidopsis thaliana</i>    | Ushimaru et al. (2006)      |
|                     | Salinity and chilling | <i>Nicotiana tabacum</i>       | Roxas et al. (2000)         |
| GST/Glutathione POD | Salinity and chilling | <i>Nicotiana tabacum</i>       | Yoshimura et al. (2004)     |
|                     | Salinity              | <i>Arabidopsis thaliana</i>    | Qi et al. (2004)            |

A chimeric gene consisting of the coding sequence for cytosolic Cu/Zn-SODs from *Oryza sativa* fused to the chloroplast transit sequence from *Arabidopsis thaliana* GR was used for generating transgenic tobacco plants. The first generation of the transgenic lines showed enhanced tolerance to salt and water stresses over the wild type, suggesting that the overexpressed Cu/Zn-SOD enhances the chloroplast antioxidant system (Badawi et al., 2004).

Transgenic *Arabidopsis* plants overexpressing Mn-SOD showed enhanced tolerance to salt stress (Wang et al., 2004). When Mn-SOD from *Nicotiana plumbaginifolia* was targeted to tobacco mitochondria, only a minor effect on ozone tolerance was observed. However, the overproduction of SOD in the chloroplasts resulted in a 3–4 fold reduction of visible ozone injury in transgenic tobacco plants (Van Camp et al., 1994). The overexpression of Fe-SOD increases  $O_2^{\cdot-}$ -scavenging capacity and thereby improves the oxidative stress tolerance of plants. Transgenic alfalfa plants transformed with *Arabidopsis* Fe-SOD with a chloroplast transit peptide showed a novel Fe-SOD in native PAGE. As no detectable difference in the pattern of primary freezing injury, as shown by vital staining, or additional accumulation of carbohydrates in field-acclimated roots of the transgenic alfalfa plants could be observed, it was suggested that Fe-SOD overexpression reduced secondary injury symptoms and thereby enhanced recovery from stresses experienced during winter (McKersie et al., 2000).

Attempts have been made to improve the stress tolerance of many crop plants by manipulating CAT genes (Mohamed et al., 2003; Nagamiya et al., 2007; Moriwaki et al., 2008; Guan et al., 2009). Salinity stress is a major limiting factor in the productivity of cereals. In an attempt to improve salt tolerance of rice, a CAT gene of *Escherichia coli*, *katE* was introduced into japonica rice cv. Nipponbare and indica rice cv., BR5. The resultant transgenic rice plants constitutively expressing *katE* were more tolerant to NaCl than wild-type plants (Nagamiya et al., 2007; Moriwaki et al., 2008). When bacterial CAT gene was overexpressed in tomato chloroplasts, the transgenic plants had increased tolerance to photo-oxidative stress imposed by drought stress or chilling stress (Mohamed et al., 2003). CAT has also been used in preventing the plant from Cd-induced oxidative stress caused by ROS. When a CAT gene from *Brassica juncea* was introduced into tobacco, wild-type plants became chlorotic and almost dead while transgenic tobacco plants still remained green and phenotypically normal under 100 mM Cd treatment (Guan et al., 2009).

APX plays an important role in scavenging ROS by degrading  $H_2O_2$  in plants. The use of APX genes for plant transformation has led to the development of transgenic plants with enhanced tolerance to oxidative stress (Slooten et al., 1995b; Wang et al., 2005b; Duan et al., 2006; Xu et al., 2008a,b). Overexpressing a cytosolic APX from *Arabidopsis thaliana* in the chloroplasts of *Nicotiana tabacum* cv. SR1 resulted in a significantly enhanced  $H_2O_2$  scavenging capacity (Slooten et al., 1995b). The different isoforms of APX in cytosol have different functional roles in rice. Transgenic lines overexpressing OsAPXb showed higher salt tolerance than OsAPXa transgenic *Arabidopsis* lines. Enhanced active oxygen scavenging system was suggested to protect plants from salt stress by equilibrating  $H_2O_2$  metabolism in these lines. The overproduction of OsAPXb enhanced and maintained the higher level of APX activity than OsAPXa in transgenic *Arabidopsis* during treatment with different concentrations of NaCl. Findings suggest that the rice cytosolic OsAPXb gene has a more functional role than OsAPXa in the improvement of salt tolerance in transgenic plants (Lu et al., 2007). Thylakoid-bound APX and peroxisomal type APX (pAPX) also play an important role in protection against various abiotic stress-induced oxidative stresses (Xu et al., 2008a,b; Sun et al., 2009). When thylakoid-bound APX gene (*LetAPX*) from tomato was overexpressed in tobacco, improved salt tolerance was observed in transgenic plants (Sun et al., 2009). Transgenic *Arabidopsis thaliana* plants carrying a pAPX gene (*HvAPXI*) from barley were found to be more tolerant to salt stress than the wild type. The salt tolerance in transgenic plants was not due to the maintenance and re-establishment of cellular ion homeostasis but by the reduction of oxidative stress injury (Xu et al., 2008a). Transgenic rice plants overexpressing *HvAPXI* under excessive Cd were significantly more tolerant to Cd stress and accumulated more Cd compared to the wild-type plants (Duan et al., 2006). *HvAPXI* also plays important roles in protection against excessive Zn-induced oxidative stress and appears to be a novel candidate gene for developing high-biomass Zn-tolerant plants for phytoremediation of Zn-polluted environments (Xu et al., 2008b). The mechanism of Zn tolerance in transgenic plants was suggested to be due to reduced oxidative stress damage.

The enzyme MDHAR is crucial for the regeneration of AsA and essential for maintaining a reduced pool of AsA. The overexpression of *MDHAR* has been shown to minimize the deleterious effects of environmental stresses (Eltayeb et al., 2007). Transgenic tobacco plants overexpressing *Arabidopsis thaliana MDHAR* gene (*AtMDAR1*) in the cytosol exhibited up to 2.1-fold higher MDAR1 activity and 2.2-fold higher level of reduced AsA compared to nontransformed control plants. The transgenic plants showed enhanced stress tolerance in terms of significantly higher net photosynthesis rates under ozone, salt, and PEG stresses and greater PSII effective quantum yield under ozone and salt stresses. Furthermore, these transgenic plants exhibited significantly lower  $H_2O_2$  level when tested under salt stress (Eltayeb et al., 2007). These results demonstrate that an overexpressed level of *MDAR* properly confers enhanced tolerance against ozone, salt, and PEG stress (Eltayeb et al., 2007).

The enzyme DHAR is assumed to be critical for AsA recycling. The manipulation of DHAR expression is important for the genetic engineering of stress-tolerant plants (Kwon et al., 2003; Ushimaru et al., 2006; Amako and Ushimaru, 2009). The expression of rice DHAR in transgenic

*Arabidopsis thaliana* enhanced resistance to salt stress (Ushimaru et al., 2006). Similarly, enhanced tolerance to NaCl and low temperature was observed when a human DHAR was overexpressed in tobacco plants. Transgenic plants showed higher DHAR and GR activities than nontransgenic plants and the ratio of AsA/DHA increased from 0.21 to 0.48, even though total AsA content was not significantly changed. When tobacco leaf discs were subjected to oxidative stress, reduction in membrane damage relative to nontransgenic plants was observed. Furthermore, transgenic plants showed enhanced tolerance to low temperature and NaCl compared to nontransgenic plants (Kwon et al., 2003). Glutathione peroxidase reduces  $H_2O_2$  to  $H_2O$  by oxidizing GSH. Transgenic tobacco seedlings overexpressing glutathione S-transferase with glutathione peroxidase activity resulted in enhanced growth under a variety of stressful conditions (Roxas et al., 2000). Similarly, the overexpression of glutathione S-transferase gene of *Suaeda salsa* enhanced the salt tolerance of transgenic *Arabidopsis* plants (Qi et al., 2004). Increased GSH-dependent peroxide scavenging and alterations in GSH and AsA metabolism was suggested to reduce abiotic stress-induced oxidative damage in glutathione S-transferase overexpressed transgenic plants (Roxas et al., 2000; Qi et al., 2004). Glutathione peroxidase-like protein overexpressed either in the cytosol or in chloroplasts of tobacco plants increased tolerance to oxidative stress caused by various stressful conditions by removing unsaturated fatty acid hydroperoxides generated in cellular membranes (Yoshimura et al., 2004).

Flavonoids are a class of plant secondary metabolites. Increased flavonoids content has been used to generate potato tubers with modified antioxidant capacities. The single-gene overexpression or the simultaneous expression of genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) resulted in a significant increase of measured phenolic acids and level of anthocyanins accompanied by decreases in starch and glucose levels in transgenic plants. The flavonoids-enriched plants showed improved antioxidant capacity, however the participation of other compounds (which are not yet recognized) was also suggested in rendering antioxidant potential to the plants (Lukaszewicz et al., 2004). In plants, the xanthophyll cycle (the reversible interconversion of two carotenoids, violaxanthin, and zeaxanthin) is a promising target for genetic engineering to enhance stress tolerance. In *Arabidopsis thaliana*, the overexpression of the *chyB* gene that encodes  $\beta$ -carotene hydroxylase, an enzyme in the zeaxanthin biosynthetic pathway, causes a specific twofold increase in the size of the xanthophyll cycle pool. The plants are more tolerant to conditions of high light and high temperature, as shown by reduced leaf necrosis, reduced production of the stress indicator anthocyanin, and reduced lipid peroxidation. Stress protection was suggested to be due to the function of zeaxanthin in preventing the oxidative damage of membranes (Davison et al., 2002). In view of the antioxidant properties of  $\alpha$ -tocopherol, it is suggested that the overexpression of  $\alpha$ -tocopherol can increase the tolerance of plants to oxidative stress caused by abiotic stresses (Liu et al., 2008). Tocopherol cyclase (VTE1, encoded by *VTE1* gene) catalyzes the penultimate step of tocopherol synthesis. Transgenic tobacco plants overexpressing VTE1 from *Arabidopsis* showed decreased lipid peroxidation, electrolyte leakage, and  $H_2O_2$  content, compared to the wild type when exposed to drought conditions (Liu et al., 2008).

Though it is possible to confer a certain degree of tolerance to a particular stress by overexpressing a single component of antioxidant defense system, only limited improvement in stress tolerance has been observed in these plants (Lee et al., 2009). As ROS detoxification system is very complex, overexpressing one enzyme may or may not change the capacity of the whole pathway. Therefore, increases in one component may not result in an overall increase in protection (Lee et al., 2009). For example, Rubio et al. (2002) found no improvement to oxidative or environmental stress tolerance in transgenic alfalfa overexpressing SOD. Similarly, rice plants overexpressing *SodCc1*, encoding Cu/Zn-SOD showed no enhanced tolerance to oxidative stress. Further, wilting assay also demonstrated no improvement in tolerance to either cold or drought (Lee et al., 2009). The expression of combinations of antioxidant enzymes in transgenic plants has been shown to have synergistic effects on stress tolerance (Tseng et al., 2008). Chinese cabbage overexpressing both SOD and CAT in cytosol shows considerable tolerance to  $SO_2$  damage (Tseng et al., 2008). Transgenic plants with an enhanced tolerance to multiple environmental stresses have been developed by overexpressing

the genes of both SOD and APX in the chloroplasts (Kwon et al., 2002; Lee et al., 2007; Lim et al., 2007; Kwak et al., 2009). Lee et al. (2007) showed that the simultaneous expression of multiple antioxidant enzymes, such as Cu/Zn-SOD, APX, and DHAR, in chloroplasts is more effective than single or double expression for developing transgenic plants with enhanced tolerance to multiple environmental stresses.

## 5.6 CONCLUSIONS AND FUTURE PROSPECTS

One of the inevitable consequences of abiotic stresses like drought, salinity, heat, chilling, heavy metal, anaerobiosis, gaseous pollutants, and UV-B radiation involves the production of ROS in different cellular compartments, such as chloroplasts, peroxisomes, and mitochondria. ROS play two divergent roles in plants: in low concentrations, they act as signaling molecules for the activation of defense responses under stresses, whereas in high concentrations, they cause exacerbating damage to cellular components. If prolonged over a certain extent, abiotic stresses, through overproduction of ROS, would result in oxidative damage to lipids, proteins, and nucleic acids, in turn causing severe damage to cell viability. The enhanced production of ROS is, however, kept under tight control by versatile and cooperative ROS-scavenging antioxidant mechanisms that modulate intracellular ROS concentration. These mechanisms can be conveniently divided in two groups, viz. nonenzymic antioxidants such as GSH, AsA, tocopherols, carotenoids, etc., and the enzymic antioxidants like CAT, POX, SOD, as well as enzymes of AsA–GSH cycle such as APX, MDHAR, DHAR, and GR. Antioxidant responses of plants not only depend on the species-inherent strategy but also on the tissue, duration, and severity of the stress period. Molecular and cellular knowledge associated with abiotic stress induced various damages, and metabolic alterations are necessary to improve abiotic stress tolerance in plants. Naturally, abiotic stress-tolerant plants provide helpful tools for such research. Enhancements in the expression of components of antioxidant defense system involved in ROS-scavenging show significant improvements in metabolic status of plants, and this strategy has been used to develop crop plants with enhanced stress tolerance. The study of the genetically transformed plants has allowed us to understand the roles of individual enzymes in metabolic regulations. However, attempts to increase abiotic stress tolerance by overexpressing one of the components of antioxidant defense system have not always been successful because it may not change the capacity of the pathway as a whole. Further, it can disturb the balanced interaction among the components. The expressions of the combination of antioxidant components have shown synergistic effects on stress tolerance and have been used frequently to produce transgenic plants with enhanced tolerance to abiotic stresses. Various abiotic stressful conditions of the environment severely limit agricultural productivity throughout the world. Therefore, future work employing biotechnological approaches is essential to produce crop plants with in-built capacity of enhanced levels of multiple abiotic stress tolerance by constitutively expressing high levels of antioxidants, for cultivation in stress-prone environments.

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# 6 Antioxidant Protection during Abiotic Stresses

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Ever since the introduction of molecular oxygen ( $O_2$ ) into our atmosphere by  $O_2$ -evolving photosynthetic organisms 2.7 billion years ago, reactive oxygen species (ROS) have been the unwelcome companions of aerobic life. In contrast to  $O_2$ , these partially reduced or activated derivatives of oxygen [singlet oxygen ( $^1O_2$ ), superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $HO\cdot$ )] are highly reactive and toxic, and can lead to the oxidative destruction of cells. Consequently, the evolution of all aerobic organisms has been dependent on the development of efficient ROS-scavenging mechanisms. In recent years, a new role for ROS has been identified: the control and regulation of biological processes, such as growth, cell cycle, programmed cell death, hormone signaling, biotic and abiotic stress responses, and development. These studies extend our understanding of ROS and suggest a dual role for ROS in plant biology as both toxic byproducts of aerobic metabolism and key regulators of growth, development, and defense pathways. How this dual role is controlled in plants is largely unknown. However, it is clear that the steady-state level of ROS in cells needs to be tightly regulated. In *Arabidopsis*, a network of more than 150 genes is involved in managing the level of ROS. This network is highly dynamic and redundant, and encodes ROS-scavenging and ROS-producing proteins. Recent studies have unraveled some of the key players in the network, but many questions related to its mode of regulation, its protective roles and its modulation of signaling networks that control growth, development and stress response remain unanswered. The hunt for ROS receptors in plants is still open. It has been suggested that plant cells sense ROS via at least three different mechanisms: (1) unidentified receptor proteins; (2) redox-sensitive transcription factors, such as NPR1 or Heat Shock Factors; and (3) direct inhibition of phosphatases by ROS. Continued genomics, proteomics and metabolomics efforts and other emerging technologies are likely to provide a more detailed picture of the networks involved in different ROS-related plant processes. Because ROS play a regulatory role

in plants response and adaptation to both biotic and abiotic stress situations, new insights into the ROS gene network might also allow the identification of genes that can ultimately be exploited to modulate ROS-related plant processes that lead to the generation of better performing crop plants. (by Frank Van Breusegem and Ron Mittle)

6.1 INTRODUCTION

Plants are frequently exposed to a plethora of unfavorable or even adverse conditions, called abiotic stresses, which lead to a series of morphological, physiological, biochemical, and molecular changes that prevent plants from reaching their full genetic potential, and hence limit the crop productivity. Abiotic stress is the principal cause of crop failure worldwide, dipping average yields for most major crops by more than 50% [1]. A common consequence of abiotic stresses is that they result, at some stage of stress exposure, in an increased production of ROS and subsequent oxidative stress. To maintain growth and productivity, plants must adapt themselves to stress conditions and set up specific protective mechanisms, including the protective mechanisms against oxidative stress. The chief aim of this chapter is to make a survey of protection by means of antioxidant systems against oxidative stress in plants during the action of various abiotic stresses.

6.2 REACTIVE OXYGEN SPECIES

Plants, being aerobic organisms, utilize molecular dioxygen (O<sub>2</sub>) as a terminal electron acceptor. The reduction of O<sub>2</sub> to H<sub>2</sub>O provides the energy that allows the impressive complexity of higher organisms on one hand, but on the other hand, its incomplete reduction produces ROS [2].

As oxidative stress together with ROS is elaborated in the chapter “Oxidative Stress in Plants: Production, Metabolism, and Biological Roles of Reactive Oxygen Species” by Tsanko Gechev and Ivan Minkov, Bulgaria, we will confine only to the compendium of the most important plant antioxidants together with their strategies in Table 6.1.

**TABLE 6.1**  
**Compendium of the Most Important Enzymatic and Nonenzymatic Antioxidants**  
**in Plants with Their Abbreviations Used in the Text**

| Antioxidant                            | Strategy                                                                                                                  |
|----------------------------------------|---------------------------------------------------------------------------------------------------------------------------|
| Superoxide dismutase (SOD)             | $2O_2^{\cdot-} + 2H^+ \Rightarrow O_2 + H_2O_2$                                                                           |
| Ascorbate peroxidase (APX)             | $H_2O_2 + 2Asc \Rightarrow 2H_2O + 2MDHA$                                                                                 |
| Monodehydroascorbate reductase (MDHAR) | $2MDHA + NAD(P)H + H^+ \Rightarrow 2Asc + NAD(P)^+$                                                                       |
| Dehydroascorbate reductase (DHAR)      | $2DHA + 2GSH \Rightarrow 2Asc + GSSG$                                                                                     |
| Glutathione reductase (GR)             | $GSSG + NAD(P)H + H^+ \Rightarrow 2GSH + NAD(P)^+$                                                                        |
| Catalase (CAT)                         | $2H_2O_2 \Rightarrow 2H_2O + O_2$                                                                                         |
| Glutathione peroxidase (GPX)           | $H_2O_2 + 2GSH \Rightarrow 2H_2O + GSSG$                                                                                  |
| Glutathione (GSH)                      | $H_2O_2 + 2GSH \Rightarrow 2H_2O + GSSG$<br>$GSH + OH^{\cdot} \Rightarrow H_2O + GS^{\cdot}$                              |
| Ascorbate (Asc)                        | $2Asc + H_2O_2 \Rightarrow 2H_2O + 2DHA$                                                                                  |
| β-carotene (β-car)                     | $\beta-car + ROO^{\cdot} \Rightarrow \beta-car^{\cdot} + ROOH$<br>$^3Chl^* + \beta-car \Rightarrow ^1Chl + ^3\beta-car^*$ |
| α-Tocopherol (α-toc)                   | $\alpha-toc + LOO^{\cdot} \Rightarrow \alpha-toc^{\cdot} + LOOH$                                                          |
| Xanthophyll cycle                      | Heat dissipation of excess energy                                                                                         |
| Flavonoids (Fl)                        | $Fl-OH + R^{\cdot} \Rightarrow Fl-O^{\cdot} + RH$                                                                         |
| Lycopene (Lyc)                         | $Lyc + O_2^{\cdot-} \Rightarrow Lyc^{\cdot-} + O_2$                                                                       |



### 6.3 DROUGHT STRESS

During drought stress, stomata are closed in order to limit water loss. In consequence, the CO<sub>2</sub> concentration is limited in the leaf mesophyll tissue. For example, the decrease of water potential to -3MPa and the relative water content to 42% caused a reduction of the maximum diurnal CO<sub>2</sub> assimilation rate by 80% in rosemary (*Rosmarinus officinalis* L.) [3]. The decrease of CO<sub>2</sub> results in an accumulation of NADPH (Photosystem I). The availability of NADP<sup>+</sup> (nicotinamide adenine dinucleotide phosphate), an electron acceptor for PSI (nicotinamide adenine dinucleotide phosphate, reduced form), is scarce. Under these conditions, O<sub>2</sub> acts as an alternate acceptor of electrons, resulting in the formation of O<sub>2</sub><sup>-</sup> [4]. Oxidative damage has been linked as the cause of plant injuries experienced during drought [5–7].

The changes in antioxidant enzyme activities depend on both severity and the duration of water shortage. GR (glutathione reductase) activity in wheat leaves was higher in drought stress than in irrigated plants [8]. On the other hand, in pea plants with a leaf water potential of about -1.3 MPa, CAT (catalase), DHAR (dehydroascorbate reductase), and GR activities decreased by 72%–85%, but APX (ascorbate peroxidase) and MDHAR (monodehydroascorbate reductase) decreased only by 15% [9]. In tobacco leaves, the CAT activity decreased, whereas the activities of APX, GR, and SOD (superoxide dismutase) were not affected significantly by water withdrawal. In the roots of the same plants, the responses were more significant: the activity of CAT, though very low, decreased, but GR and APX elevated in drought conditions [10].

Transient increases in SOD, APX, and GR activities occurred at 6 and 12 days of drought, respectively in both fescue (*Festuca arundinacea* L.) and Kentucky bluegrass (*Poa pratensis* L.); however, the activities of all three enzymes decreased with extended periods of drought [11]. For example, in *Vigna* seedlings, CAT activity and ascorbate content decreased with increasing water stress (-0.5 to -1.5 MPa) [12].

In many grass species, a rise in the total glutathione pool and a depletion of ascorbate was observed during drought stress [13]. The concentration of  $\alpha$ -tocopherol increased 15-fold and that of carotenoids by approximately 26% in water-stressed rosemary (*Rosmarinus officinalis* L.) [3]. Similarly, in sage (*Salvia officinalis* L.) and lemon balm (*Melissa officinalis* L.), significant increases in  $\alpha$ -tocopherol occurred, as well as the ascorbate level in chloroplasts increased [3]. In *Phyllyrea angustifolia* L., the amounts of  $\alpha$ -tocopherol increased up to fourfold and those of zeaxanthin, a pigment of the protective xanthophyll cycle, increased up to threefold. While  $\alpha$ -tocopherol increased further in severe drought, zeaxanthin and  $\beta$ -carotene decreased; during recovery, the  $\beta$ -carotene level increased slowly and the  $\alpha$ -tocopherol level decreased sharply [14].

### 6.4 WATERLOGGING

During waterlogging or submergence by excessive water, plants are exposed to a reduction in oxygen supply because of the slow diffusion rate of oxygen in water and its limited solubility [15]. Oxygen deprivation stress in plant cells is distinguished by three physiologically different states: transient hypoxia, anoxia, and, eventually, reoxygenation.

Hypoxic tissues exhibit enhanced mitochondria-dependent superoxide formation via xanthine oxidase, lipoyxygenase action on membrane lipids, and finally lipolytic acylhydrolase-catalyzed liberation of free fatty acids, which underpins a burst in lipid peroxidation or return to normoxia. Anaerobic tissues have a very high redox potential and the soil environment surrounding the roots contains highly reduced forms of metal ions, such as Fe<sup>2+</sup>, which can readily reduce atmospheric oxygen to a superoxide. Therefore, in the interim between the return to high oxygen partial pressures and the reactivation of the mitochondrial electron transport system, conditions are ideal for the activation of oxygen [16]. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of barley [17,18] and in wheat roots [19]. The presence of H<sub>2</sub>O<sub>2</sub> in the apoplast and in association with the plasma membrane has been visualized by transmission electron

microscopy under hypoxic conditions in four plant species [20]. In these experiments,  $H_2O_2$  was probably of enzymatic origin, considering the low oxygen concentration in the system and the positive effects of the various inhibitors of  $H_2O_2$ -producing enzymes. Indirect evidence of ROS formation (i.e., lipid peroxidation products) under low oxygen has been detected [21–25].

It was observed in a study with soybean roots that a short anoxic stress (1–2 h) increased the potential for superoxide production [26]. With longer durations of anoxia (3–5 h), the roots developed an increased ability to cope with oxygen-free radicals, and therefore exhibited less postanoxic injury. The increase in SOD activity has been reported in rhizomes of flooded *Iris pseudacorus* [27]. An increase in the total SOD activity has also been detected in wheat roots under anoxia but not under hypoxia. The degree of increase positively correlated with the duration of anoxia [19]. The induction of SOD activity by 40%–60% has been shown under hypoxia both in the roots and leaves of barley [17,18]. Short-term flooding led to an increase in the activities of GR, APX, CAT, and SOD in maize leaves, while under prolonged flooding, inhibition of these enzymes occurred [23]. In submerged seedlings (i.e., under hypoxic conditions), the activities of antioxidant enzymes were lower compared with air-germinated controls. On the other hand, the induction of enzymes involved in the ascorbate-dependent antioxidant system (APX, MDHAR, and DHAR) has been shown for anaerobically germinated rice seedlings after transfer to air [28]. Acclimation to anoxia has been shown to be dependent, at least partly, on peroxidases, which have been up-regulated by anoxic stress [29]. In rice seedlings, SOD activities responded nonsignificantly to submergence, while CAT activity increased upon the readmission of oxygen [30]. Investigations involving 11 species differing in their tolerance to anoxia have revealed an increase in MDHAR and/or DHAR in the anoxia-tolerant plants after several days of anoxic treatment. On the contrary, the activities were very low or without any changes in the sensitive plants [31]. A slight decrease in MDHAR, DHAR, and GR activities occurred in wheat seedlings under hypoxia, while anoxia caused a significant increase in the reduced ascorbate and glutathione [32].

The imposition of anoxia and subsequent reoxygenation caused a decrease both in the content of ascorbate and in its reduction state in the roots of cereals and the rhizomes of *Iris* spp. [33]. During long-term anoxic stress, tocopherol contents in the rhizomes of two iris species—highly anoxia-tolerant *Iris pseudacorus* and anoxia-sensitive *I. germanica*—have been compared. Tocopherols ( $\alpha$ - and  $\beta$ -) were identified in both iris species,  $\beta$ -tocopherol being the predominant tocopherol isomer, especially in rhizomes of *I. germanica*, which also possessed a markedly higher total tocopherol content than *I. pseudacorus*. Anoxia caused a decrease in the contents of tocopherol isomers in both iris species [33].

## 6.5 HIGH TEMPERATURE

In nature, plants are subjected to changes of temperature, both during the shift in season and, more abruptly, over the course of individual days. The temperature of an individual plant cell can change much more suddenly and rapidly than other factors causing stress, for example, water or salt levels [34].

The leaf copes with increasing temperature by opening stomata; the transpiration flow cools down the tissue [35]. However, when the temperature reaches high values, stomata close. The characteristic response to high temperature is the heat shock, characterized by the rapid induction of heat shock proteins (HSPs) that primarily function as molecular chaperones to ensure the correct function of many cellular proteins under conditions of elevated temperature [36]. HSP 70 was identified as a sensor of leaf temperature [37].

The photochemical efficiency of PSII decreases at elevated temperatures, indicating increased stress [38,39]. An intimate relationship appears to exist between oxidative stress and the heat shock response [40–42]. For example, tobacco seedlings showed a significant increase in  $H_2O_2$  after exposure to 40°C for 1 h [43]. Heat stress was shown to cause impairments in mitochondrial functions that resulted in the induction of oxidative damage [34,44]. Heat shock leading to a

programmed cell death in plants was shown to be associated with an enhanced production of ROS and the activation of the oxidative burst [36,45].

The steady-state transcript and protein level of many ROS-scavenging enzymes were found to be elevated by heat stress [36,45–49]. SOD activity increased up to ninefold in the heat-tolerant tomato cultivars but decreased in the heat-sensitive cultivar, whereas CAT and APX activities increased significantly in all cultivars [46]. In rice seedlings, the level of APX activity was higher in heated seedlings, whereas CAT activity was decreased. There was no significant difference in SOD activity between heated and unheated seedlings [36,47]. Both transgenic apple and tomato plants overexpressing cytosolic APX had improved resistance to heat stress [50,51].

## 6.6 LOW TEMPERATURE

Suboptimal growth temperatures provoke oxidative stress in plants. The amount of  $H_2O_2$  detected in leaves increases as the growth temperature declines [52–56]. The damage caused to mature and developing leaves by low-temperature stress manifests primarily in the chloroplasts, leading to the inhibition of photosynthesis and premature senescence [57–59]. Studies on the relationships between  $CO_2$  assimilation, photosynthetic electron transport, and antioxidant enzyme activities in field-grown maize suggested that the donation of electrons to oxygen by the photosynthetic electron transport chain was increased by plant growth at low temperatures [60]. However, the  $H_2O_2$  content in maize leaves was increased by growth at suboptimal temperatures, but was independent of irradiance [61,62]. This suggests that photosynthesis is not directly responsible for the low-temperature-induced increase in the foliar  $H_2O_2$  level [62].

There is evidence that the chilling tolerance of plants is correlated with increasing amounts of antioxidants and increasing activity of free-radical-scavenging enzymes. Exposure to low temperatures caused an increase in CAT and GR activities [63,64]. Similarly, high APX, MDHAR, DHAR, GR, and SOD activities and enhancement of the ascorbate and  $\alpha$ -tocopherol pools were observed in field-grown maize during periods when the plants were exposed to chilling [60]. Decreased CAT, APX, and MDHAR activities were found to be associated with chilling sensitivity during the early stages of development of four inbred lines of *Zea mays* [56,65,66]. While the activities of GR, APX, and SOD were markedly increased due to chilling, the activity of CAT changed to a lesser extent and was dependent on the maize genotype analyzed [67]. The response of antioxidant enzymes depended on the duration of chilling stress. GR activity increased with the increasing length of the cold period in maize, while SOD activity changes depended also on the maize genotype [68].

Higher GR activities and higher ascorbate level were found in leaves of the chilling-tolerant *Zea diploperennis* than in the chilling-sensitive *Zea mays* [69]. A chilling-tolerant maize genotype has been shown to contain higher amounts of both  $\alpha$ -tocopherol and glutathione and higher GR activity than a chilling-sensitive maize genotype [70]. Chilling-induced enhancement of the glutathione pool and constitutively higher GR activities were found to contribute to the chilling tolerance in *Zea mays* [71].

It is known that ascorbate regenerates tocopherols from their radical forms [72]. However, the artificially increased ascorbate content in maize leaves did not improve the preservation of endogenous tocopherol during high light and chilling stress [73].

## 6.7 EXPOSURE TO HIGH LIGHT INTENSITIES

Chloroplasts are considered to be the most powerful source of ROS in plants even under normal conditions [74]. Under excess light conditions ( $\geq 1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), chloroplasts accept more energy than they are able to utilize. Thus, the electron transport chain in chloroplasts becomes overreduced and enzymatic processes for  $CO_2$  fixation become the rate-limiting step. As a result, photosynthesis produces more NADPH and ATP than is necessary; consequently, a lack of respective acceptors occurs. Such accumulation of redox and energy equivalents reduces the plastoquinone pool and/or inhibits the water-splitting complex, inevitably leading

to PSII inactivation, so-called photoinhibition [75–79]. Exposure to high light intensities leads to higher intracellular levels of ROS due to increased rates of  $O_2$  photoreduction in chloroplasts and increased flux of  $H_2O_2$  in peroxisomes via photorespiration [80,81]. Nonfunctional PSII is also known to generate  $^1O_2$  in high quantities [82].

In a broad range of plant species, a strong correlation has been reported between the intensity of the light environment and foliar activities of a number of antioxidant enzymes and contents of ascorbate and glutathione [83–85]. It has been suggested that enhancements in the activities of activated-oxygen-scavenging enzymes during high light stress occur mostly in the chloroplasts [43]. This could be supported by the fact that tobacco plants overexpressing chloroplast Cu/Zn SOD were less sensitive to photoinhibition than nontransgenic controls [86]. However, the exposure of *Arabidopsis* plants to light stress triggered ROS production not only in the chloroplast but also in the cytosol [87].

The rapid increase in SOD activity and the gradual increase in the activities of APX, GR, DHAR, and MDHAR were observed in whole cells in *Begonia x erythrophylla* when exposed to high light [88]. On the other hand, it has been reported that both the synthesis and degradation of CAT are light sensitive [89], and the light impairment of CAT activity was described [90]. The decline in CAT activity combined with the rapid oxidation of both the ascorbate and glutathione pools, and the rapid increase in SOD activity that would have resulted in the rapid conversion of  $O_2^{\cdot-}$  to  $H_2O_2$ , could have resulted in the rapid increase of cellular  $H_2O_2$ . A reduction in CAT activity could also lead to lowered breakdown of the  $H_2O_2$  formed as a result of photorespiration and mitochondrial electron transport [88].

As regards low molecular antioxidant response to excess light, several deficient mutants were investigated. Leaf disks of the *Arabidopsis thaliana* mutant *vte1*, which is deficient in tocopherol cyclase activity resulting in a complete lack of tocopherols, were suddenly exposed to an intense white light, due to which chlorophylls and lipids were rapidly photooxidized, but no such alterations were observed in tocopherol-containing wild-type leaf disks [91]. The *vte2* mutant, which accumulates only 10%–30% of the wild-type level of ascorbate [92,93], is also sensitive to high light and shows bleaching when transferred from low light to high light [94,95].

## 6.8 UV RADIATION

UV radiation can be divided into three classes according to its energy: UV-A, UV-B, and UV-C. UV-A (315–400 nm) is not attenuated by atmospheric ozone, and this less damaging radiation is an important photomorphogenic signal in plant development [96]. UV-B (280–315 nm) is strongly absorbed by atmospheric ozone; however, incident UV-B radiation in low layers in the troposphere is increasing due to the reduction in the stratospheric ozone concentration [97]. Highly energetic UV-C (wavelengths  $\leq 280$  nm) is strongly absorbed by oxygen and ozone in the stratosphere such that none of this sterilizing radiation is present in terrestrial sunlight.

Many of the effects of UV-B on plants are likely to be more or less a direct result of cellular damage caused by aberrant photoproducts in macromolecules such as DNA [98] and proteins [99,100]. Not least, UV-B radiation induces the production of ROS [101]. The most common protective mechanism against the potentially damaging effect of the irradiation is the biosynthesis of UV-absorbing compounds [102], which include different classes of flavonoids. Flavonoids accumulate in the vacuoles of epidermal cells where they attenuate the UV component of sunlight with minimal absorption of photosynthetically active radiation [103,104]. As was mentioned above, flavonoids are strong antioxidants, and most flavonoids outperform even well-known antioxidants, such as ascorbate and  $\alpha$ -tocopherol, in in vitro assay [105].

Exposure of *Nicotiana plumbaginifolia* to UV-B strongly increased CAT and GPX (glutathione peroxidase) mRNA, but APX and SOD transcript levels remained unaltered [106]. The SOD activity increased and the CAT activity decreased both in green and in etiolated barley seedlings [107]. In cucumber (*Cucumis sativus* L.) cotyledons, UV-B enhanced the activity of SOD peroxidase as well as the ascorbate content, but decreased the level of  $\alpha$ -tocopherol [108].

Transgenic plants overexpressing enzymes involved in ROS scavenging are very often more tolerant to UV-B radiation. Examples of such plants are the methyl viologen-resistant *Arabidopsis* mutant (*rcd1-2*), with enhanced activity of plastidic Cu/Zn SOD and stromal APX [109], and tomato (*Lycopersicon esculentum*) overexpressing cytosolic APX [110].

*Arabidopsis* mutant (*tt5*), which is flavonoid deficient, synthesized new isoforms of APX after UV-B irradiation [111]. Rao et al. suggested that UV-B exposure preferentially induces peroxidase-related enzymes.

As mentioned above, UV-C is not present in terrestrial sunlight; however, it has been employed experimentally to induce strong oxidative stress. SOD, APX, CAT, and GR activities markedly increased in UV-C irradiated bean (*Phaseolus vulgaris* L.) cotyledons. With prolonged irradiation, the activities of SOD, APX, and GR started to decrease, but CAT activity henceforward increased [112]. In these cotyledons,  $\beta$ -carotene and zeaxanthin increased, but ratios between reduced and oxidized ascorbate, as well as between reduced and oxidized glutathione sharply decreased [113].

## 6.9 SALINITY STRESS

Salinity affects almost all aspects of plant development: germination, vegetative growth, or reproduction. Soil salinity imposes ion toxicity, osmotic stress, nutrient deficiency, and oxidative stress in plants. Sodium accumulation in cell walls can rapidly lead to osmotic stress and cell death [114]. High salt concentrations in soil impose osmotic stress and, thus, limit water uptake from soil. Ion toxicity is the result of replacement of  $K^+$  by  $Na^+$  in biochemical reactions, and  $Na^+$  and  $Cl^-$  induced conformational changes in proteins. For several enzymes,  $K^+$  acts as a cofactor, which cannot be substituted by  $Na^+$ . High  $K^+$  concentration is also required for the binding of tRNA to the ribosomes, and thus protein synthesis is affected [115]. Ion toxicity causes metabolic imbalances, which in turn leads to oxidative stress [116].

Increases in activities of SOD, APX, and GR under salinity stress have been observed in many species [117–119]. However, this statement is not user type: while both CAT and APX activities declined and that of SOD increased in tobacco leaves, activities of GR and APX increased while the SOD activity decreased in the roots of the same plants [10].

Relatively higher Cu/Zn SOD, Fe SOD, APX, and GR activities in the chloroplastic fraction and Mn SOD in the mitochondrial fraction in tolerant wheat genotypes have also been reported [120]. NaCl enhanced both mRNA expression and activities of Mn SOD, APX, GR, and MDHAR in tolerant pea cv. Granada, whereas a significant change was neither observed in mRNA levels nor in the activities of those enzymes in salinity-sensitive cv. Chillis [121]. Similarly, in chloroplasts from tolerant pea plants, the NaCl stress induced a significant increase in Cu/Zn SOD and APX activities as well as in the ascorbate content, while in chloroplasts from sensitive plants, NaCl evoked increases in the  $H_2O_2$  content and lipid peroxidation and no changes were observed in the enzymatic activities [122].

Transgenic tobacco overexpressing GR did show increased levels of lipid peroxidation under salinity stress [123]. On the other hand, overexpression of APX in tobacco chloroplasts enhanced the tolerance to salt stress [124]. Also, transgenic rice overexpressing Mn SOD was more tolerant against salinity stress compared to wild type [125].

As a protection against salt stress, plants accumulate various osmolytes for maintaining ionic homeostasis, for example, proline, mannitol, pinnitol, ononitol, and sorbitol in cytosol. Simultaneously, these organic osmolytes have also been proved to be antioxidants: the presence of mannitol in chloroplasts can protect plants against oxidative damage by hydroxyl radicals [126]. Similarly, exogenous proline decreased the rate of lipid peroxidation, the content of superoxide radical, and, consequently, SOD activity (almost fivefold), and increased the content of chlorophylls (*a* and *b*) in the leaves of halophyte *Mesembryanthemum crystallinum* [127].

## 6.10 HEAVY METALS

As a consequence of industrial development, the environment has been increasingly polluted with heavy metals [128]. Their toxicity comprises the inactivation of biomolecules either by blocking essential functional groups or by displacing essential metal ions [129]. The binding of metals to sulfhydryl groups in proteins is important, leading to an inhibition of activity or the disruption of a structure [130]. In addition, an excess of heavy metals may stimulate the formation of ROS [131,132], and oxidative stress caused by exposure to heavy metals has been reported many times [133–137]. Certain heavy metals like Cu and Fe exert toxicity by their participation in redox cycles, producing hydroxyl radicals in Fenton reaction [138]. On the contrary, Cd is not redox active and, thus, unable to participate directly in Fenton-type reactions [139], but can indirectly initiate the production of different ROS by unknown mechanisms, giving rise to an oxidative burst [140–143].

The changes in activities of antioxidant enzymes depend on the duration, the type of metal, and the strength of stress treatment. In sunflower (*Helianthus annuus* L.), CAT activity decreased to 24% and 70% of the control activities in Cu- and Cd-treated leaf disks, respectively. APX activity behaved similarly. SOD activity decreased to 50% and 27% after Fe and Cd treatments, respectively [134].

In pea plant leaves treated by 50  $\mu$ M CdCl<sub>2</sub>, CAT and SOD activities decreased, but GR activity did not show significant changes. A strong reduction of chloroplastic and cytosolic Cu/Zn SODs by Cd was found, and Fe SOD to a lesser extent, while Mn SOD was the most resistant isoform at 40  $\mu$ M Cd. To determine if the observed reduction in the SOD activities was due to a direct interaction of Cd with the enzymes, commercial Cu/Zn SOD and pea leaf extracts were incubated with 100  $\mu$ M Cd for 24 and 72 h, respectively. In these conditions, no effect by Cd on the Cu/Zn SOD activity was detected even after 72 h incubation. CAT isoenzymes responded differentially, the most acidic isoforms being the most sensitive to the Cd treatment [139].

Roots and leaves of pea plants exposed to 4 and 40  $\mu$ M of Cd for 7 days in hydroponic culture responded differently. Cd-induced enhancement in SOD activity was higher at 40  $\mu$ M than at 4  $\mu$ M concentration in leaves. While CAT activity prominently increased in leaves both at 4 and 40  $\mu$ M Cd, APX showed maximum stimulation at 40  $\mu$ M Cd in roots. Enhancement in GR activity was also more at 40  $\mu$ M than at 4  $\mu$ M Cd in roots. While GPX activity decreased in roots and remained almost unmodified in leaves, glutathione S-transferase showed pronounced stimulation in both roots and leaves of pea plants exposed to 40  $\mu$ M Cd [144].

After prolonged Pb stress, the activation of SOD and CAT was induced in extracts from pea roots [145]. With increased duration of treatment and Pb<sup>2+</sup> concentration, the activities of SOD, CAT, and APX increased as well. The root cells redox state of glutathione, expressed as the ratio of GSH/GSSG, dropped proportionally to Pb stress intensity [146]. Similarly, in maize (*Zea mays* L., cv. Samodek) callus cultures exposed for a long period (22 months) to Pb (0.5 mM PbCl<sub>2</sub>), APX and GR activities increased [147]. The increased activity of GPX in pea in the presence of copper has been presumed to be due to its role in metal detoxification [148].

Besides being radical scavengers, flavonoids are also able to function as chelators for metals [149]. In cell cultures of *Ginkgo biloba*, flavonoids accumulated up to 12-fold in response to Cu treatment [150]. Similarly, in callus cultures of the legume plant *Ononis arvensis*, flavonoid level increased after both Cu and Cd treatments [151].

## 6.11 AIR POLLUTANTS

O<sub>3</sub> is an important protective component against UV-C radiation in the stratosphere, but it is one of the most notorious air pollutants in the troposphere. It enters the plant cell through the stomata and breaks down in the apoplast to produce different ROS [152]. The flux of O<sub>3</sub> in the leaf interior is controlled almost entirely by stomatal conductance [153]. O<sub>3</sub> is a strong oxidant, and ROS are first observed in guard cell chloroplasts and membranes, and later they spread to the neighboring cells [154].

The increase in the ROS level in guard cells limits further diffusion of ozone into the leaf by promoting stomatal closure. Acute exposure to O<sub>3</sub> often results in foliar lesion, such as chlorosis and necrosis [155], whereas accelerated leaf senescence has been observed for chronic exposure to low ozone levels [156].

O<sub>3</sub> is considered to react first with oxidizable constituents of the apoplast, most probably through the action of apoplastic NADPH oxidase [157]. Subsequently, if O<sub>3</sub> and/or its reactive products escape interception, they react with the components of the plasmalemma and cytosol [158–160].

The first detoxifying barrier, which can be considered as constitutive since it represents the antioxidant system found in the cell (apoplast + symplast) at the time of ozone attack, will scavenge ozone and its derivatives. This system is tightly linked to the level of ascorbate, and especially apoplastic ascorbate, which was primarily proposed as a good indicator for ozone tolerance. For example, the reaction of O<sub>3</sub> with apoplastic ascorbate in the leaves of *Vicia faba* was potentially sufficient to intercept a substantial proportion (30%–40%) of O<sub>3</sub> entering the plant under environmentally relevant conditions [160]. Similarly, when in an ozone-sensitive genotype of *Raphanus sativus* L. the ascorbate content was increased by supplying the biosynthetic precursor L-galactono-1,4-lactone, tolerance to O<sub>3</sub> was improved [161]. The *Arabidopsis* mutant lacking completely cytosolic DHAR—a key component of the ascorbate recycling system—was highly O<sub>3</sub> sensitive [162].

However, elevated apoplastic ascorbate levels did not seem to be sufficient to explain plant tolerance [163–165]. In fact, the antioxidative capacity of the apoplastic space is considered much weaker than that of the cell interior [166], and it can be overwhelmed by a very important ozone flux [165]. An inductive response, triggered by the ROS, takes place allowing exchanges of antioxidants between the symplastic detoxification system and the apoplast. Although the resulting increased level of symplastic ascorbate could serve as a predictor of the resistance to ozone attack [167], other molecules and the cellular ability to regenerate antioxidants could be more relevant [164,165,168].

For example, *Nicotiana tabacum* plants overexpressing chloroplastic Cu/Zn SOD or GR did not show enhanced ozone tolerance [169,170], whereas tobacco overexpressing cytosolic Cu/Zn SOD showed less leaf injury than wild-type plants (5% versus 25%, respectively) upon exposure to near-ambient ozone regimes [171]. SOD levels increased twofold in poplar hybrid after 90 min of ozone exposure (180 ppb O<sub>3</sub>). In the same poplar, reduction of photosynthesis induced by high O<sub>3</sub> concentrations was preceded by a significant increase in the foliar glutathione content, but almost 50% of the total glutathione became oxidized under these conditions [172]. Nevertheless, elevated foliar activities of glutathione synthase or GR alone were not sufficient to improve the tolerance of hybrid poplar to acute ozone stress [173].

## 6.12 CONCLUSIONS

The production of ROS during environmental stress is one of the main causes for decreases in productivity, injury, and death that accompany these stresses in plants [174]. Current data suggest certain correlation between plant antioxidants and plant stress tolerances; however, it is impossible to draw general conclusions. Ambiguity of some results is probably caused by different designs of experiments and different ages of plants when they are exposed to a particular stress. Also, the impact of a stress on a plant differs; hence, the plant responses are different as well. The reaction of a plant cell to adverse conditions is probably cross talk among responses of individual organelles and compartments. Moreover, we have to consider also other important regulators of plant responses to stresses, namely, hormones. They mutually regulate stress response.

In nature, the antioxidant response depends not only on the stress length and intensity but also on stress combination—plants are rarely exposed to one stress only *au naturel*. Slow progress in breeding for abiotic stress-tolerant crops can be attributed to the poor understanding of the molecular mechanisms associated with tolerance against combined stresses. Further research is necessary to better understand the role of antioxidants in combination with various abiotic stresses.

During the past decade, applications of molecular tools, such as gene knockout or transgenic approaches, have improved our understanding of plant stress defense. There is no doubt that the utilization of transgenic plants grown under either single stress but better under-stress combinations that occur in nature will uncover many important developments, which will contribute in increasing crop production.

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# 7 Biochemical Mechanisms for the Maintenance of Oxidative Stress under Control in Plants

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Sergio A. Guerrero, and Alberto A. Iglesias*

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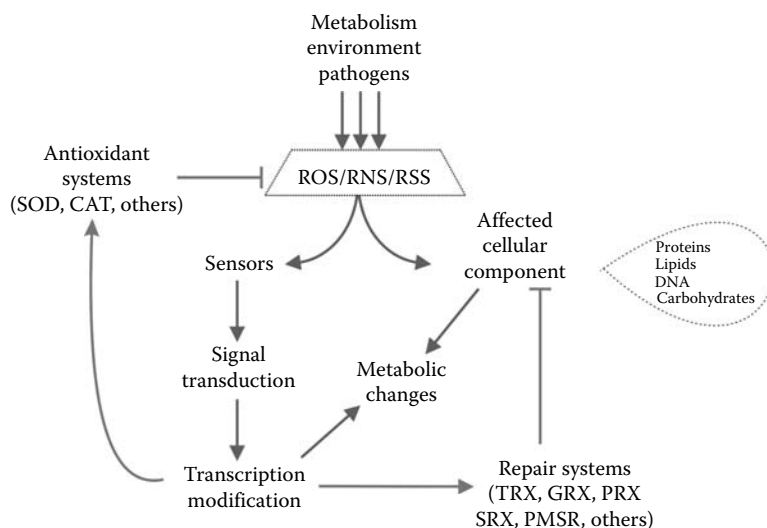
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## 7.1 OXIDATIVE REACTIVE SPECIES PARADIGM: REDOX SIGNAL TRANSDUCTION VERSUS ANTIOXIDANTS STRESS RESPONSE

It is believed that over 2.2 billion years ago, the presence of O<sub>2</sub> in the Earth's atmosphere was originated due to the evolution of cyanobacteria photosynthetic activity, which used the sun's energy and water to produce sugars with the release of O<sub>2</sub>. Since then, several organisms began to evolve an antioxidant defense that gives them the capacity to tolerate O<sub>2</sub> and use it for metabolic transformation and biosynthesis (Halliwell 2006, Slesak et al. 2007). Considering the relationship between aerobic organisms and O<sub>2</sub>, it is remarkable that this molecule can be both beneficial and damaging. Beneficial for the reason that O<sub>2</sub> is essential as electron exchanger for an efficient respiration and photosynthetic activity and it is largely used for signal transduction; while damaging because O<sub>2</sub> can cause dysfunction on cell components by irreversible modifications on DNA, proteins, sugars, and lipids. Hence, there is a balance/imbalance between both sides, which is critical for the cell functionality and survival (Slesak et al. 2007).

During evolution in an aerobic atmosphere, which unavoidably exposes organisms to an oxidative environment, plants and all other aerobic life-forms evolved different antioxidative systems to protect cell components. Consequently, a delicate homeostasis between oxidative and reductive reactions is maintained inside cells, which is critical in the use of O<sub>2</sub> in metabolic pathways. The presence of intracellular oxidative and reducing species determine a transitory redox environment, defined as the summation of the reduction potential and reducing capacity of the linked redox couples occurring inside the cell (Schafer and Buettner 2001). When this redox environment suffers, an imbalance and reduced or oxidized species are favored, cells are exposed to a redox stress. Both oxidative and reductive stress can trigger redox cascades that bring changes in the oxidized/reduced status of biomolecules. Changes in the cellular redox environment can alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and even regulation of the cell cycle. Thus, the redox environment might determine if a cell will proliferate, differentiate, or perish (Moller et al. 2007, Neill et al. 2002). Figure 7.1 schematizes how different agents can create stress conditions and also molecular damages, the whole situation challenging the cell to trigger defense mechanisms. On the other hand, Figure 7.2 shows a simplified scenario of different metabolic routes related with the flux of redox equivalents operating under physiological conditions as well as to coping situations of oxidative stress.

The use of O<sub>2</sub> in metabolic reactions through electron transport chains such as in photosynthesis and respiration produces NADPH and energy (ATP), but also generates different reactive oxygen species (ROS) as by-products (Apel and Hirt 2004, Buchanan and Balmer 2005) (Figure 7.1). ROS are different oxygen-derived species; for example, free radicals containing one or more unpaired electrons such as superoxide anion radical (O<sub>2</sub><sup>•−</sup>), hydroxyl radical (•OH), per-hydroxyl radical (HO<sub>2</sub><sup>•</sup>), singlet oxygen (<sup>1</sup>O<sub>2</sub>), and nonradical derivatives such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Foyer et al. 2009).

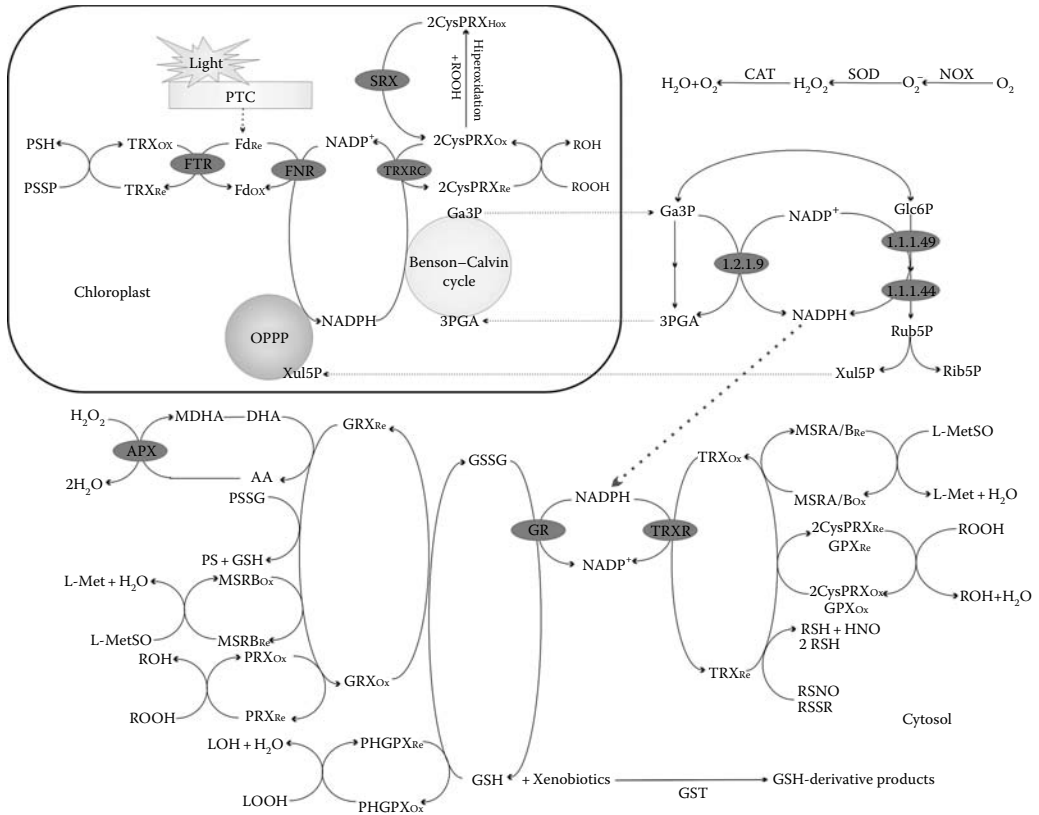


**FIGURE 7.1** Redox homeostasis, redox signaling, and oxidative stress. The scheme shows a probable progression of the cellular response elicited by synthesis of oxidant species. Different modifications triggered by oxidants are interrelated in order to increase the activity of antioxidant systems which might struggle to return the system to homeostasis.

In addition, plants may also be exposed to reactive nitrogen species (RNS) and reactive sulfur species (RSS) (Figure 7.1). The major RNS in the cell is nitric oxide ( $\cdot\text{NO}$ ) that can react with  $\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ , oxidized glutathione (GSSG), and transition metals generating peroxynitrite ( $\text{ONOO}^-$ ), nitrosoglutathione (GSNO), and metal-NO adducts, respectively (Neill et al. 2008). Other components like ozone ( $\text{O}_3$ ), nitric dioxide ( $\text{NO}_2$ ), and sulfur dioxide ( $\text{SO}_2$ ), which are major air pollutants, can cause oxidative modifications as well (Moller et al. 2007).

To struggle with this different oxidative species, aerobic organism has developed an antioxidant network composed by many antioxidant systems (Figure 7.1). The term “antioxidant” can be considered to describe any compound capable of quenching ROS without itself undergoing conversion to a destructive radical (Noctor and Foyer 1998). The antioxidant network is composed by numerous proteins, enzymes, and metabolites that act in synchrony to ameliorate oxidative stress situations. Some of the proteins/enzymes acting in plant antioxidative networks are catalase (CAT), peroxiredoxin (PRX), thioredoxin (TRX), glutaredoxin (GRX), and sulfiredoxin (SRX) whereas main metabolites involved include ascorbate, glutathione (GSH), tocopherol, and NAD(P)H. The enzymatic and nonenzymatic antioxidant components work together to form different groups of antioxidant systems.

Plants, as photosynthetic aerobic organisms, are highly exposed to oxidative conditions. Moreover, due to their sessile lifestyle, plants are unconditionally exposed to a range of biotic and abiotic deleterious stresses that can trigger an oxidative burst. Besides, these oxidative species can function initially as signal transducers (Figure 7.1). The situation of oxidative stress depends on the sort of ROS that is produced, the concentration and the site where molecules are generated, their interaction with other molecules present in the organism, as well as the developmental stage and the past of the cell (Moller et al. 2007). Accumulation of  $\text{H}_2\text{O}_2$ , for example, is perceived by the plant as a signal of environmental change. As a diffusible signal-transducer molecule,  $\text{H}_2\text{O}_2$  alerts metabolism to the presence of both biotic and abiotic threats (Noctor and Foyer 1998). If the plant response is not enough to overcome this threat, the oxidative stress is established. Comprehension of occurrence and function of antioxidant metabolic routes is of high interest to understand (1) how plants detoxify oxidative species, (2) how they rearrange the whole metabolism under oxidative conditions, (3) how signal transduction pathways operate in response to oxidative stress, and (4) how cells repair macromolecules after oxidative damage.



**FIGURE 7.2** Cross talk between antioxidant systems. The scheme shows fluxes between main redox components in plants.  $O_2$ : molecular oxygen,  $O_2^-$ : superoxide anion,  $H_2O_2$ : hydrogen peroxide,  $H_2O$ : water, NOX: NADPH oxidase SOD: superoxide dismutase, CAT: catalase, PTC: photosynthetic transport chain, Fd: ferredoxin, FNR: ferredoxin-NADP oxido-reductase, FTR: ferredoxin-thioredoxin oxide-reductase, TRX: thioredoxin, PSH: thiol-protein, PSSP: disulfide-protein, TRXRC: thioredoxin reductase C, 2CysPRX: typical two cysteine peroxidoreductase, ROOH: peroxide, ROH: alcohol, SRX: sulfiredoxin, Ga3P: glyceraldehyde-3-phosphate, 3PGA: 3-phosphoglycerate, (1.2.1.9): non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, Glc6P: glucose-6-phosphate, (1.1.1.49): Glc6P dehydrogenase, (1.1.1.44): 6-phosphogluconate dehydrogenase, Rub5P: ribulose-5-phosphate, Rib5P: ribose-5-phosphate, Xul5P: xylulose-5-phosphate, OPPP: oxidative pentose phosphate pathway, GR: glutathione reductase, GSSG: oxidized glutathione, GSH: reduced glutathione, GRX: glutaredoxin, DHA: dehydroascorbate, AA: ascorbic acid, APX: ascorbate peroxidase, MDHA: monodehydroascorbate, PSSG: glutathionyl-protein, MSRB: B-type methionine sulfoxide reductase, L-MetSO: L-methionine sulfoxide, L-Met: L-methionine, PRX: peroxidoreductase, PHGPX: phospholipid hydroperoxide glutathione peroxidase, LOOH: lipid hydroperoxide, LOH: lipid alcohol, TRXR: thioredoxin reductase, MSRA: A-type methionine sulfoxide reductase, GPX: glutathione peroxidase, RSNO: S-nitrosothiols, RSSR: low molecular weight disulfide, RSH: low molecular weight thiol, HNO: nitroxyl, GST: glutathione-S-transferase.

## 7.2 OXYGEN, NITROGEN, AND SULFUR REACTIVE SPECIES: VERSATILITY OF OXIDANT MOLECULES

Oxidant species include a great number of reactive molecules chemically derived from partial reduction of oxygen, nitrogen, or sulfur (Giles et al. 2001, Nordberg and Arner 2001). Production of these molecules is a natural, constant, and inevitable process linked to cellular metabolism and to the interaction of the cell with its environment (Apel and Hirt 2004) (Figure 7.1). Some of these molecules (singlet oxygen, hydroxyl radical, peroxynitrite, or sulfenic acid) are extremely

reactive, while others (superoxide anion and hydrogen peroxide) are reactive but in a smaller degree (Giles and Jacob 2002, Nordberg and Arner 2001, Patel et al. 1999). Oxidant species have the potential to cause oxidative damage by reacting with many biomolecules (Figure 7.1), being able not only to modify their structures but also to produce a loss in their specific biological functions (Apel and Hirt 2004, Friguet 2006). Recently, it has become apparent that oxidant species have roles as signaling molecules (Figure 7.1) that contribute to the control of plant development, in the sensing of external environment and in the defense against pathogens (Forman et al. 2004, Foyer and Noctor 2005, Pauly et al. 2006, Vranova et al. 2002).

## 7.2.1 REACTIVE OXYGEN SPECIES

ROS include a number of chemically reactive molecules derived from oxygen, such as oxygen ions, free radicals, and peroxides, both inorganic and organic (Forman et al. 2004); all of them being highly reactive due to the presence of electrons at unpaired valence shell. ROS are generated as natural products of the normal metabolism of oxygen and have important roles in cell signaling and oxidative stress (Hancock 1997, Kotchoni and Gachomo 2006, Neill et al. 2002). Next, the most common intracellular forms of ROS are described.

### 7.2.1.1 Singlet Oxygen

Singlet oxygen ( $^1\text{O}_2$ , diamagnetic form of molecular oxygen), can be generated by an input of energy that rearranges electrons in its valence shell (Halliwell 2006). Because of the differences in their electron shells, singlet and triplet oxygen ( $^3\text{O}_2$ , most stable form of molecular oxygen) differ in their chemical properties (Halliwell 2006). In  $^1\text{O}_2$ , the spin restriction is removed and the oxidizing ability greatly increased, being able to directly oxidize proteins, DNA, and lipids (Triantaphylides and Havaux 2009). Note that  $^1\text{O}_2$  is not a free radical, since it has no unpaired electron. In plants, insufficient energy dissipation during photosynthesis can lead to the formation of a chlorophyll triplet state that can transfer its excitation energy to ground-state  $\text{O}_2$  to form  $^1\text{O}_2$  (Halliwell 2006, Triantaphylides and Havaux 2009). This can oxidize chloroplast molecules and also trigger cell death. Plants have molecules that can quench the  $^1\text{O}_2$ , such as carotenoids, tocopherols, plastoquinones, ascorbate, vitamin B6, and flavonoids (Triantaphylides and Havaux 2009). The  $^1\text{O}_2$  is also sometimes used as a signaling molecule, having a crucial role in plant responses to light (Halliwell 2006, Triantaphylides and Havaux 2009).

### 7.2.1.2 Superoxide Anion

Superoxide anion ( $\text{O}_2^{\cdot-}$ ) is the product of the one-electron reduction of  $\text{O}_2$ , which occurs widely in nature (Vranova et al. 2002). With one unpaired electron,  $\text{O}_2^{\cdot-}$  is a free radical that is paramagnetic (as is the case for  $\text{O}_2$ ) (Apel and Hirt 2004, Forman et al. 2004); even so, it is not highly reactive, lacking the ability to penetrate lipid membranes and therefore remaining trapped within the compartment where it is generated (Nordberg and Arner 2001). Formation of  $\text{O}_2^{\cdot-}$  takes place spontaneously, especially in electron rich aerobic environments in the vicinity of the mitochondria inner membrane (Nordberg and Arner 2001). It can also be produced endogenously by flavoenzymes, such as NADPH oxidase, lipoxygenase, and cyclooxygenase (Kotchoni and Gachomo 2006).  $\text{O}_2^{\cdot-}$  has important roles both in redox signaling response to different stimulus and in defense against pathogens (Kotchoni and Gachomo 2006). Because  $\text{O}_2^{\cdot-}$  is toxic, almost all living organisms contain isoforms of superoxide dismutase (SOD) (Apel and Hirt 2004), an extremely efficient enzyme that catalyzes dismutation of two molecules of  $\text{O}_2^{\cdot-}$  into hydrogen peroxide and  $\text{O}_2$  (Apel and Hirt 2004).

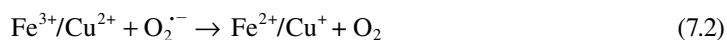
### 7.2.1.3 Hydrogen Peroxide

Even when hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is not a free radical, its damaging capacity is important after its ability to penetrate biological membranes (Apel and Hirt 2004, Bienert et al. 2006, Neill et al. 2002). It plays a role as an intermediate in the formation of other ROS, including HOCl (hypochlorous acid)

and  $\cdot\text{OH}$  (formed via oxidation of transition metals, see below) (Nordberg and Arner 2001).  $\text{H}_2\text{O}_2$  is moderately reactive and relatively stable (half-life of  $10^{-3}$  s), exhibiting remarkable diffusion capacity (Neill et al. 2002). It can function as an intracellular signaling molecule inducing a wide range of molecular, biochemical, and physiological responses within cells (Hancock 1997, Neill et al. 2002).  $\text{H}_2\text{O}_2$  can inactivate enzymes through oxidation of their thiol groups. Generation of  $\text{H}_2\text{O}_2$  in plants is induced following exposure to a wide variety of abiotic and biotic stimuli, like extreme temperatures, UV irradiation, excess of excitation energy, exposure to ozone, phytohormones (i.e., ABA), dehydration, wounding, and pathogen attack (Apel and Hirt 2004, Kotchoni and Gachomo 2006, Neill et al. 2002). Potential sources of  $\text{H}_2\text{O}_2$  include  $\text{O}_2^{\cdot-}$  dismutation by SOD, NADPH oxidase, cell-wall peroxidase, amino oxidase, oxalate oxidase, and flavin-containing oxidase (Neill et al. 2002).  $\text{H}_2\text{O}_2$  is removed by at least four antioxidant enzyme systems, namely, catalases, glutathione peroxidases, ascorbate peroxidases (APXs), and peroxiredoxins (PRXs) (Forman et al. 2004, Noctor and Foyer 1998).

#### 7.2.1.4 Hydroxyl Radical

Hydroxyl radical ( $\cdot\text{OH}$ ) has a very short half-life (approx.  $10^{-9}$  s), and is an extremely dangerous compound to organisms because of its strong reactivity toward biomolecules (Halliwell 2006, Nordberg and Arner 2001). This radical species is capable of damaging biological systems to higher extent than any other ROS. Unlike  $\text{O}_2^{\cdot-}$ , which can be detoxified by SOD, the  $\cdot\text{OH}$  cannot be eliminated enzymatically (Halliwell 2006). As the diffusion rate is slower than the half-life, this species will react with any oxidizable compound in its vicinity. It can damage almost all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (peroxidation), and amino acids (e.g., conversion of phenylalanine to *m*- and *o*-tyrosine) (Halliwell 2006, Triantaphylides and Havaux 2009, Vranova et al. 2002). Cells utilize compounds as glutathione or ascorbate to protect important structures from  $\cdot\text{OH}$  (Noctor and Foyer 1998). This radical is formed from  $\text{H}_2\text{O}_2$  in a reaction catalyzed by metal ions ( $\text{Fe}^{2+}$  or  $\text{Cu}^+$ ), often bound in complex with different proteins or other molecules [This is known as the Fenton reaction (7.1) (Nordberg and Arner 2001)].  $\text{O}_2^{\cdot-}$  also plays an important role in connection with the Fenton reaction by recycling metal ions (7.2) (Nordberg and Arner 2001, Vranova et al. 2002). The sum of these reactions is the Haber–Weiss reaction, which illustrates the important role played by transition metals in the formation of  $\cdot\text{OH}$  (Vranova et al. 2002).



### 7.2.2 REACTIVE NITROGEN SPECIES

RNS are a family of reactive molecules derived from  $\cdot\text{NO}$  produced via the enzymatic activity of inducible  $\cdot\text{NO}$  synthase (NOS) (Besson-Bard et al. 2008). They act together with ROS to damage cells, causing the so-called nitrosative stress. RNS are also continuously produced in plants as by-products of aerobic metabolism or in response to stress (Besson-Bard et al. 2008, Shapiro 2005). Nitration of proteins and lipids represent key biologically relevant redox signaling and injury events (Neill et al. 2002). These processes involve the participation of  $\cdot\text{NO}$ -derived species such as peroxyntirite ( $\text{ONOO}^-$ ) and nitrogen dioxide ( $\cdot\text{NO}_2$ ) generated during oxidative and nitrosative stress (Droge 2002, Squadrito and Pryor 1998). Many biomolecules are oxidized and/or nitrated by RNS, including protein tyrosine residues, thiols, and unsaturated fatty acids (Droge 2002, Squadrito and Pryor 1998).

#### 7.2.2.1 Nitric Oxide

Like  $\text{O}_2^{\cdot-}$ ,  $\cdot\text{NO}$  does not directly react with most biomolecules despite its unpaired electron (Halliwell 2006, Patel et al. 1999). It easily reacts with other free radicals (i.e., peroxy and alkyl radicals), mainly generating less reactive species, thus functioning as a free-radical scavenger

[i.e.,  $\cdot\text{NO}$  has been shown to inhibit lipid peroxidation in cell membranes] (Patel et al. 1999). However, if  $\text{O}_2^{\cdot-}$  is produced in large amounts in parallel with  $\cdot\text{NO}$ , the two species react to give peroxynitrite ( $\text{OONO}^-$ ), which is highly cytotoxic (Nordberg and Arner 2001). In plants,  $\cdot\text{NO}$  can be produced by any of four routes: (1) L-arginine-dependent NOS (although this pathway has been reported in plants, neither gene, nor cDNA, nor protein related with NOS has been isolated to date.) (Corpas et al. 2004, 2006, Valderrama et al. 2007), (2) by plasma membrane-bound nitrate reductase (Corpas 2004), (3) by mitochondrial electron transport chain (Besson-Bard et al. 2008), or (4) by nonenzymatic reactions (Besson-Bard et al. 2008).

A main physiological function involves  $\cdot\text{NO}$  as an intracellular messenger, stimulating guanylate cyclase, and protein kinases (Besson-Bard et al. 2008, Shapiro 2005). It is a signaling molecule that acts mainly against oxidative stress and also plays a role in plant-pathogen interactions (Besson-Bard et al. 2008, Shapiro 2005).  $\cdot\text{NO}$  has the ability to cross cell membranes and can thereby transmit signals to other cells (Patel et al. 1999). A biologically important reaction of  $\cdot\text{NO}$  is *S*-nitrosylation, converting thiol groups (including cysteine residues in proteins) in *S*-nitrosothiols (RSNO) (Forman et al. 2004, Squadrito and Pryor 1998). *S*-nitrosylation is a mechanism for dynamic, posttranslational regulation of most or all major classes of proteins (Giustarini et al. 2004). Excessive production of  $\cdot\text{NO}$  is counteracted by its conjugation with glutathione that results in the *S*-nitrosoglutathione adduct (GSNO), which in turn can be cleaved directly by TRX systems, consuming NADPH and liberating GSH and  $\cdot\text{NO}$  (Giustarini et al. 2004, Nordberg and Arner 2001). The total effect of  $\cdot\text{NO}$  on cells redox status is clearly multifaceted; in many aspects, it functions as an antioxidant rather than an oxidant.

#### 7.2.2.2 Peroxynitrite

The nonradical, oxidant, and nitrating agent  $\text{ONOO}^-$  is able to react with diverse biomolecules in one or two-electron reactions, thus damaging a wide number of cell components, including DNA and proteins (Landino 2008, Nordberg and Arner 2001). Formation of  $\text{ONOO}^-$  *in vivo* has been described to occur by reaction of  $\text{O}_2^{\cdot-}$  with  $\cdot\text{NO}$  (Besson-Bard et al. 2008, Halliwell 2006, Nordberg and Arner 2001). Also,  $\text{ONOO}^-$  readily reacts with  $\text{CO}_2$  to form highly reactive nitroso peroxocarbonate ( $\text{ONOOCO}_2^{\cdot-}$ ), which can generate  $\cdot\text{NO}_2$  and  $\cdot\text{CO}_3^-$  (carbonate radical), two potent oxidizing agents (Halliwell 2006, Nordberg and Arner 2001, Valderrama et al. 2007). At the physiological pH,  $\text{ONOO}^-$  rapidly protonates to peroxynitrous acid ( $\text{ONOOH}$ ) (Halliwell 2006).  $\text{ONOO}^-$  can directly damage proteins, lipids, and DNA (Landino 2008, Valderrama et al. 2007); but it can also cause damage by undergoing homolytic fission to give noxious products,  $\cdot\text{OH}$  and  $\cdot\text{NO}_2$ , or rearrange to nitrate ( $\text{NO}_3^-$ ) (Squadrito and Pryor 1998).  $\text{ONOO}^-$ , directly or via its reaction products, modifies tyrosine and cysteine residues in different proteins, being able to inactivate them (Besson-Bard et al. 2008, Landino 2008).

#### 7.2.2.3 S-Nitrosothiols

*S*-nitrosothiols (RSNO) may cause many of the biological effects of  $\cdot\text{NO}$ , mainly producing *S*-nitrosylation of proteins (Forman et al. 2004). They serve as donors of nitrosonium ion ( $\text{NO}^+$ ) and  $\cdot\text{NO}$ , which proceed as signaling molecules in plant cells (Zhang and Hogg 2005). The addition of a nitroso group to the sulfur atom of cysteine residues in proteins is known as *S*-nitrosation or *S*-nitrosylation (Forman et al. 2004, Zhang and Hogg 2005). This reversible process is a form of protein posttranslational modification, which can be regulated through mechanisms analogous to phosphorylation (Giustarini et al. 2004, Zhang and Hogg 2005). Generation of *S*-nitrosothiol adducts leads to changes in activity, interaction, or subcellular location of target proteins (Giustarini et al. 2004, Zhang and Hogg 2005).

### 7.2.3 REACTIVE SULFUR SPECIES

ROS and RNS are primary oxidizing agents generated after oxidative stress (Apel and Hirt 2004, Forman et al. 2004). There is a second generation of reactive species that possess different spectrum

of activity respect to redox reactions and biological targets (Giles et al. 2001). Thus, while sulfur is usually considered part of cellular antioxidant systems, rising evidences support the formation of RSS (with suppressor properties resembling ROS and RNS) under conditions of oxidative stress (Giles and Jacob 2002, Jacob et al. 2004). Thiols, as well as disulfides, are easily oxidized to species with sulfur in higher oxidation states (Jacob et al. 2004). Such agents include sulfenyl radicals ( $\text{RS}\cdot$ ), disulfides (RSSR), sulfenic acids ( $\text{RSO}\cdot$ ), disulfide-*S*-oxides [ $\text{RS(O)SR}$ ], and disulfide-*S*-dioxides [ $\text{RS(O}_2\text{)SR}$ ] (Giles and Jacob 2002). These species modulate the redox status of biological thiols by reaction with low molecular weight thiols and thiol moieties present in proteins. Examples include evidences that  $\text{RS(O)SR}$  are able to inactivate glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, and also that RSS induce zinc release from zinc-finger domains (Giles and Jacob 2002, Jacob et al. 2004).

### 7.3 MODIFICATION OF CELLULAR COMPONENTS: OXIDATIVE DAMAGE AND SIGNAL PERCEPTION

As illustrated by Figure 7.1, exposure to physiological concentrations of reactive oxidant species induces metabolic changes leading to cell responses such as repair, adaptation, or transformation (Forman et al. 2004). Reactive species are able to cause damage, and thereby represent potentially toxic and mutagenic agents (Smirnoff 2005), being targets for such damages all major groups of biomolecules: lipids, proteins, and nucleic acids (Halliwell 2006). The oxidative reactions are mainly irreversible and may induce (in some cases) specific repair mechanisms (Moller et al. 2007). Despite their deleterious effects, reactive species also play a central role both as indicators of oxidative stress and as signaling molecules. In contrast to oxidative stress, redox signaling always involves responses specific to oxidation–reduction reactions following a reversible mechanism (Forman et al. 2004), having reactive species as second messengers involved in signal transduction.

#### 7.3.1 EFFECTS ON DNA, LIPIDS, AND PROTEINS

##### 7.3.1.1 DNA

Reactive species have been shown to be mutagenic agents by chemically modifying DNA in different ways, mainly on the nucleotide bases (Banerjee 2008). A number of alterations include cleavage of DNA, DNA–protein cross-link, oxidation of guanine to 8-hydroxyguanine, and conjugation with polyunsaturated fatty acids (PUFA) (Moller et al. 2007, Mueller and Berger 2009). In addition to mutations, oxidative DNA modifications can lead to changes in the methylation of cytosines, which is important for regulating gene expression (Moller et al. 2007). These modifications are due to reactions with ROS, especially  $\cdot\text{OH}$  (the most reactive) and  $^1\text{O}_2$  (primarily attacking guanine) (Britt 1996, Nordberg and Arner 2001). [Contrarily,  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  are not reactive at all with DNA]. If repairing systems are not able to immediately regenerate intact DNA, a mutation will result after erroneous base pairing during replication (Nordberg and Arner 2001). A number of mechanisms are available for repairing DNA damage both, in the nucleus and in the mitochondria (Kimura et al. 2004). These include direct reversion of the damage, replacement of the base, and replacement of the whole nucleotide (Banerjee 2008, Britt 1996, Roldan-Arjona and Ariza 2009).

##### 7.3.1.2 Lipids

PUFAs, such as linoleic acid (18:2) and linolenic acid (18:3) are the major fatty acids in plant membranes (Smirnoff 2005). After their multiple double bonds, PUFA are excellent targets for free radical (particularly  $^1\text{O}_2$  and  $\cdot\text{OH}$ ) attack, giving rise to a complex mixture of lipid hydroperoxides (LOOH) (Moller et al. 2007, Mueller and Berger 2009). Lipid peroxidation is probably the most explored area of research when it refers to ROS. Extensive PUFA peroxidation decreases the fluidity of the membrane, increases leakiness, and causes secondary damage to membrane proteins

(Moller et al. 2007). Several products of PUFA peroxidation can form conjugates with DNA and proteins (Banerjee 2008, Nordberg and Arner 2001). In plant cells, some PUFA oxidation products function as secondary messengers either directly or after enzymatic modification (Moller et al. 2007). Enzymatic systems involving LOOH detoxification include a member of glutathione peroxidase family, the phospholipid-hydroperoxide glutathione peroxidase (Banerjee 2008). This enzyme can directly reduce lipid hydroperoxides, utilizing reduced glutathione as a reductive equivalent donor (Smirnoff 2005).

### 7.3.1.3 Proteins

Protein oxidation is defined here as a covalent modification of a protein induced by ROS, RNS, or by-products of oxidative stress (Banerjee 2008). Protein oxidation is a widespread reaction and is often used as a diagnostic marker for oxidative stress (Moller and Kristensen 2004, 2006). Most types of protein oxidation are essentially irreversible, whereas a few involving sulfur-containing amino acids are reversible (Forman et al. 2004, Halliwell 2006). Reaction of proteins with oxidant species generates modifications of the types: (1) side chains oxidation [mainly cysteine (Kiley and Storz 2004), methionine (Tarrago et al. 2009), and tryptophan oxidation (Moller and Kristensen 2006)]; (2) carbonylation (Banerjee 2008); (3) nitrosylation (Landino 2008); and (4) interaction with products of PUFA oxidation (Moller et al. 2007). These modifications can reach different degrees, producing from less active enzymes to denatured (nonfunctional) proteins.

The oxidation of the thiol group in cysteine can achieve different species, depending on the concentration and type of the reactive oxidant species and how many cysteinyl moieties are implicated. Formation of a disulfide ( $R_1-S-S-R_2$ , cystine) between two cysteine residues can be caused by several different ROS, this being an important mechanism for metabolic redox regulation (Balmer et al. 2004, Buchanan and Balmer 2005). Intra- or intermolecular disulfide bonds can be formed between thiol side chains and the reduced form can be regenerated (reconversion of cystine to cysteine) by TRX (Balmer et al. 2004, Montrichard et al. 2009) or GRX (Lillig et al. 2008, Rouhiet et al. 2008) systems. A large number of potential TRX-regulated proteins have been identified in different cellular compartments, including cytosol, chloroplasts, and mitochondria (Montrichard et al. 2009). Another type of cysteine modification is oxidation of the thiol group to higher levels, via cysteine sulfenic acid ( $R-SOH$ ), followed by cysteine sulfinic acid ( $R-SO_2H$ ). These oxidation states can be enzymatically reverted via action of SRX followed by TRX or GSH, and are probably involved in signaling pathways (Liu et al. 2006, Rey et al. 2007). The highest level of cysteine oxidation, cysteic acid ( $R-SO_3H$ ), appears to be irreversible and damaging (Kiley and Storz 2004). Cysteine can also form mixed disulfides primarily with glutathione and this might serve to protect the cysteine group against further oxidation (Rouhiet et al. 2008). Mixed disulfides can be reduced by GRXs and glutathione (Rouhiet et al. 2008).

Other amino acids can also be target of redox modification in proteins. The oxidation of methionine to methionine sulfoxide (MetSO) is another reversible modification (Banerjee 2008). The methionine sulfoxidation is reverted in a reaction catalyzed by the methionine sulfoxide reductase using TRX or GRX as the reductant (Banerjee 2008). It has been suggested that some peripheral methionine residues act as endogenous antioxidants, protecting the active site and other sensitive domains in the protein while helping to remove ROS (Petropoulos and Friguet 2006, Rouhiet et al. 2008). It has been proposed that reversible methionine sulfoxidation could constitute a key regulatory mechanism in cells (Rouhiet et al. 2008). Further oxidation of methionine to methionine sulfone (MetSO<sub>2</sub>) appears to be irreversible and destructive to the protein (Banerjee 2008).

Other two redox modifications operating for proteins are nitrosylation and carbonylation. The former is the covalent attachment of the messenger  $\cdot NO$  to the thiol moiety in cysteine, being a posttranslational modification that potentially regulates the function of proteins (Besson-Bard et al. 2008, Lamattina et al. 2003). Protein (and glutathione) thiols can react with  $\cdot NO$  derivatives to produce a range of products including disulfides, sulfenic, sulfinic and sulfonic acids, as well as S-nitrosothiols (Besson-Bard et al. 2008, Dalle-Donne et al. 2007, Shapiro 2005). Carbonylation



is an oxidative protein modification, coursing irreversibly and with derivative products commonly found in cells (Moller et al. 2007). The oxidation of a number of amino acids particularly arginine, histidine, lysine, proline, threonine, and triptophan renders free carbonyl groups (Banerjee 2008). Carbonyl derivatives can react with other side chain groups generating protein–protein cross-linking, or with other biomolecules (such as DNA and PUFA), thus modifying their biological properties (Banerjee 2008, Cattaruzza and Hecker 2008).

### 7.3.2 PROTEIN RECOVERY, REPLACEMENT, OR REMOVAL

One oxidized protein will be repaired or degraded according to the degree and type (reversible or irreversible) of chemical modification that it underwent (Friguet 2006, Moller et al. 2007). Oxidations able to be reverted include some of the modifications on cysteine and methionine (Banerjee 2008). In this way, the TRX system has been implicated in the reduction of both disulfide bridges and cystein sulfenic acid (Schurmann and Jacquot 2000, Smirnov 2005) whereas glutathione-dependent GRXs reduce both disulfide bridges and low molecular weight mixed disulfides such as glutathione adducts (Smirnov 2005). It has been shown that the oxidation of cysteine and methionine residues on proteins could contribute to the impairment of protein function and in the loss of their activity (Moller et al. 2007). In fact, methionine residues in proteins can be oxidized to methionine-S and -R sulfoxide diastereoisomeric forms, which can be catalytically reverted by peptide methionine sulfoxide reductase (MSR) enzymes MSRA and MSRB, respectively. In some cases, the latter reversion allows recovery of functionality to the modified protein (Banerjee 2008, Boschi-Muller et al. 2005, Tarrago et al. 2009). MSR enzymes were described as a very important repair system of oxidized proteins, hence contributing to regulation of the cellular redox homeostasis (Moskovitz 2005, Tarrago et al. 2009).

Oxidized proteins generally exhibit less activity and thermostability, mainly exposing their hydrophobic amino acids to the surface (Moller et al. 2007). In addition, protein damage can also be consequence of protein adducts formation with lipid peroxidation derivatives (Moller et al. 2007) and oxidation by glycation (sometimes called nonenzymatic glycosylation, which is the result of sugar bonding, such as fructose or glucose, to a protein or lipid molecule without the controlling action of an enzyme). The latter products lead to the formation of glycosylic adducts such as pentosidine or carboxy-methyl-lysine (McPherson et al. 1988).

Oxidized proteins are degraded relatively quick, probably because a change in conformation exposes more hydrophobic residues, which are better recognized by proteases (Starke-Reed and Oliver 1989). However, massive protein damage can lead to the formation of toxic aggregates; which are not only resistant to proteolysis, but can inhibit the ability of proteases to break down other oxidized proteins (Costa et al. 2007). Accumulation of oxidized protein aggregates is a hallmark of cellular aging (Friguet 2006), and it was proposed that they are a consequence of increased oxidative damage and/or of decreased degradation and repair. In the cytosol and the nucleus, the proteasome has been described as the main intracellular proteolytic pathway implicated in oxidized polypeptides degradation, and the general turnover of proteins (Moller et al. 2007). Otherwise, the Lon protease has been shown to selectively degrade oxidized proteins within the mitochondrial matrix (Friguet et al. 2008, Moller et al. 2007).

### 7.3.3 PROTEINS INVOLVED IN REACTIVE SPECIES PERCEPTION

Traditionally, proteins participating in the perception of reactive species and signal transduction have been characterized as polypeptides exhibiting a highly sensitive reversible oxidation, such as thiol/disulfide exchange (Bindoli et al. 2008, Kiley and Storz 2004). Given the reversible nature of most oxidized forms, it has been suggested that thiol modification can play roles in signal transduction that are similar to protein phosphorylation/dephosphorylation (Bindoli et al. 2008). There are several examples of proteins whose activities are modulated by thiol oxidation and reduction, but

sensors must have some specific characteristic enabling them to propagate this signal. A classical example is the OxyR transcription factor (Wang et al. 2008), which up-regulates peroxide defenses in *Escherichia coli* and a variety of other bacteria. OxyR contains two critical cysteines that are oxidized to form an intramolecular disulfide bond when cells encounter peroxide stress (Kiley and Storz 2004). Disulfide bond formation is associated with a conformational change that alters OxyR binding to DNA and allows the protein to activate the transcription of genes encoding enzymes that destroy  $H_2O_2$ , such as catalase and the alkylhydroperoxide reductase. Once concentration of  $H_2O_2$  is decreased, OxyR is reduced to reset the system (Banerjee 2008).

A wide variety of biotic and abiotic factors can induce the production of diverse reactive oxidant species. Therefore, it could be expected that perception of the oxidative signal or stress could involve different cellular components. In plants, the knowledge (at the molecular level) of oxidative signal transduction pathways, including its perception, is far from complete. Still, studies with redox-regulated proteins and proteomic analysis have provided some clues. It is known that oxidative signal-inducible I kinase (OXI1) and mitogen-activated protein kinases (MAPK) are activated by exogenous  $H_2O_2$ , but little is known about proteins that directly react with  $H_2O_2$  (Hancock et al. 2005). A proteomic approach with iodoacetamide-based fluorescence tagging of proteins in conjunction with mass spectrometric analysis was used to identify potential targets of  $H_2O_2$  in the cytosol of *Arabidopsis thaliana*. By this procedure, it was identified the cytosolic phosphorylating glyceraldehyde-3-phosphate dehydrogenase (Ga3PDHase, EC 1.2.1.12) as the most prominent modified protein.

Studies *in vitro* have shown that *Arabidopsis* Ga3PDHase is inactivated by oxidation with  $H_2O_2$ , GSSG, and GSNO; the process being reverted by dithiothreitol (DTT) and GSH (Hancock et al. 2005, Holtgreffe et al. 2008). We have kinetically characterized the oxidation of Ga3PDHase from wheat (*Triticum aestivum*) with different reactive oxidant species. Interestingly, oxidation of the enzyme with  $H_2O_2$  was 54- or 670-fold higher than with GSNO or GSSG, respectively. On the other hand, important intracellular reducing systems, such as those involving TRX or GRX, were effective for reverting damages caused by oxidants on Ga3PDHase (Piattoni, Guerrero and Iglesias, unpublished results). The possibility of reversion was limited by the degree of the enzyme oxidation, because extensive oxidative damage caused the irreversible formation of protein aggregates. After proteomic studies, it was proposed that Ga3PDHase could be involved in  $H_2O_2$  perception (Hancock et al. 2005, 2006), which is in agreement with the above experiments developed in our laboratory suggesting a physiological redox regulation (thus with sensing of  $H_2O_2$ ) of Ga3PDHase. As a whole, current information reported in plants accords with that from animals, where it was demonstrated that Ga3PDHase is a multifunctional protein, with many roles besides its enzymatic activity in glycolysis (Katoh et al. 2006, Kimura et al. 2004, Sirover 1999). Effectively, the enzyme participates in nuclear events including gene transcription, RNA transport, DNA replication, and initiation of apoptotic cell death upon nitrosylation (Azam et al. 2008, Hara et al. 2005, Katoh et al. 2006, Nakajima et al. 2007, Sen et al. 2008, Sirover 1997).

Although irreversible protein oxidation spawns loss of native structure and functional activity, recent reports demonstrated that the induced structural changes confer, to certain polypeptides, capacities to act as oxidative stress sensors. In this way, oxidized proteins become involved in several signal transduction routes (e.g., apoptosis activation), or they display “nonconventional” enzymatic activities (now acting as chaperones or transcriptional factors). In plants, as well as in mammals and some bacteria, an example of the latter is the over-oxidation of 2Cys typical peroxiredoxin (2CysPRX) (Dietz et al. 2006). 2CysPRX are peroxidases lacking prosthetic groups that mediate in the defense against oxidative stress by reducing  $H_2O_2$  and alkyl hydroperoxides (Smirnov 2005). This enzymatic activity relies on the high reactivity of two conserved cysteines, whose modification entails not only the usual thiol/disulfide exchange, but also irreversible oxidation states of the sulfur atom (to sulfinic or sulfonic acid) (Banerjee 2008, Hall et al. 2009). These changes may induce a series of posttranslational modifications, such as phosphorylation and acetylation, which contribute

to formation of high molecular mass quaternary structures with chaperone-like activity (Aran et al. 2009, Barranco-Medina et al. 2009), that further protect thermal denaturalization and act as protein activity modulator (Barranco-Medina et al. 2009, Hall et al. 2009).

## 7.4 REDUCING POWER GENERATION SYSTEMS IN PLANTS

Inside cells, the reducing power required for all detoxifying and regenerative systems derives, directly or indirectly, from NADPH or from ferredoxin. Plants exhibit some important characteristics: (1) cells are highly compartmentalized, with the occurrence of plastids and (2) the existence of two different kinds of tissues, with distinctive metabolic scenarios (photosynthetic or heterotrophic). The latter implies differences in the routes operating to produce NADPH, which are schematized in Figure 7.2.

### 7.4.1 GENERATION OF NADPH IN PLASTIDS

In plants, there are different types of plastids: those present in photosynthetic tissues, namely, chloroplasts, and those present in non-photosynthetic tissues, like amyloplasts, leucoplasts, and chromoplasts (Hudák 1997). Diversity in plastids includes specialized functions and metabolic capacities. Even more, chloroplast metabolism differs during the light or dark period. In higher plants, the production of NADPH in plastids is associated with NADP<sup>+</sup> reduction in the light (photoreduction) by the photosynthetic electron flow, or generation by the oxidative pentose phosphate pathway (OPPP) in the dark or in non-photosynthetic tissues.

During the light period, chloroplasts have an active photosynthetic activity holding NADPH as one key metabolite. Using the sun's energy, the photosynthetic electron transport chain is able to catalyze electrons transfer from water to NADP<sup>+</sup> ( $E'_{O_2/H_2O} = 815 \text{ mV}$  and  $E'_{NADP^+/NADPH} = -340 \text{ mV}$ , where  $E'$  is the midpoint oxidation-reduction potential relative to the standard hydrogen electrode at pH 7.0), which is driven by two successive photochemical reactions involving photosystem II (PSII) and photosystem I (PSI). The two photosystems are connected via an electron transport chain, which includes an integral membrane complex, the cytochrome  $b_6f$  complex. Finally, reduced ferredoxin (Fd) reduces NADP<sup>+</sup> to NADPH via an enzyme known as ferredoxin-NADP<sup>+</sup> oxidoreductase. Electron transfer from water to NADP<sup>+</sup> is coupled to proton translocation across thylakoid membranes that generate the electrochemical potential gradient driving to ATP synthesis (Bowyer and Leegood 1997).

During the dark period (as well in non-photosynthetic tissues), NADPH production inside plastids is directly linked to the OPPP (Figure 7.2) by oxidation, interconversion, and rearrangement of sugar-phosphates pools metabolites (Brownleader et al. 1997). There are two phases: (i) one oxidative (phase 1), where via two consecutive reactions glucose-6-phosphate (Glc6P) is converted to ribulose-5-phosphate (Rub5P) with the net production of NADPH and (ii) a non-oxidative phase (phase 2), involving the interconversion of pentose-P, hexose-P, and triose-P intermediaries to regenerate Glc6P in the cycle and connect it with glycolysis. In contrast to animal and yeast cells, plants have a duplicate OPPP, with one complete pathway inside the chloroplast and another one incomplete in the cytosol (which will be discussed later) (Anderson and Advani 1970, Bailey-Serres et al. 1992, Brownleader et al. 1997, Eicks et al. 2002, Kruger and von Schaewen 2003, Schnarrenberger et al. 1973, 1995). Despite the physical separation, both pathways are interconnected by specific solute transporters present in the chloroplast envelope (Eicks et al. 2002, Neuhaus and Emes 2000, Weber et al. 2005). The first reaction of the pathway is the oxidation of Glc6P by one specific dehydrogenase (Glc6PDHase, EC 1.1.1.49) to form 6-phosphoglucono- $\delta$ -lactone and NADPH. The lactone is hydrolyzed by a specific lactonase to yield the acid 6-phosphogluconate (6PG), which then undergoes oxidation and decarboxylation by a specific dehydrogenase (6PGDHase, EC 1.1.1.44) to form Rub5P and NADPH (Nelson and Cox 2004). The second phase consists of a reversible set of interconversions between phosphorylated 3-, 4-, 5-, 6-, and 7-carbon sugars, involving the enzymes

ribose-5-P isomerase (Rib5P isomerase), Rub5P 3-epimerase, transaldolase (TA), and transketolase (TK) (Debnam and Emes 1999).

With the exception of TA, the other reversible enzyme reactions are amphibolic and also play part in the Benson–Calvin cycle (Debnam and Emes 1999). Oxidation of Glc6P by Glc6PDHase is a strategic control point, to regulate the substrate partitioning between glycolysis and the OPPP. Detailed studies carried out on chloroplastidic Glc6PDHase evidenced that its activity is under coarse and fine regulatory control. The enzyme is affected by the NADPH/NADP<sup>+</sup> ratio, pH, Mg<sup>2+</sup>, and levels of Glc6P (Brownleader et al. 1997). Furthermore, the enzyme is activated in the dark (low NADPH/NADP<sup>+</sup>) by a redox mechanism involving TRX (Buchanan 1980, Scheibe 1991, Wenderoth et al. 1997). The activity of several chloroplast enzymes is known to be regulated by reversible thiol–disulfide interchange (Buchanan 1980, Scheibe 1991). During photosynthetic electron transport in the light, covalent redox modification mediated by the ferredoxin–TRX system leads to reductive activation of several stromal target enzymes: fructose-1,6-bisphosphatase, NADP-malate dehydrogenase, phosphoribulokinase, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, and others. Chloroplastidic Glc6PDHase is regulated in an opposite way by the same system, the enzyme being inactive in its reduced state (light period) (Wenderoth et al. 1997, Wendt et al. 1999). This regulation prevents futile cycles, like simultaneous carbohydrate synthesis in the Benson–Calvin cycle and catabolism by the OPPP. Thus, in accordance with its physiological role in chloroplasts, Glc6PDHase is active only during the dark phase, when NADPH supply by the photosynthetic electron flow ceases (Wenderoth et al. 1997).

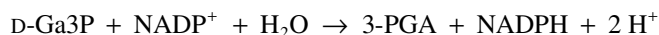
#### 7.4.2 GENERATION OF NADPH IN THE CYTOSOL OF PLANT CELLS

In the cytosol of plant cells, production of NADPH is more complicated to understand than that reported in animals and yeasts. The extent to which the OPPP operates in the cytosol of higher plants is questionable. Both Glc6PDHase (Schnarrenberger et al. 1973, 1975) and 6PGDHase (Bailey-Serres and Nguyen 1992, Bailey-Serres et al. 1992) are found in the cytosol of many plant cells. However, the organization of the reversible non-oxidative phase of the pathway is far less clear. Recent studies support the absence of Rub5P 3-epimerase, Rib5P isomerase, TK and TA in the cytosol of spinach, pea, and maize leaf cells (Debnam and Emes 1999, Schnarrenberger et al. 1995). Concurrent results were reported by Kruger and von Schaewen (2003), who analyzed the complete genome from *A. thaliana* looking for the predicted subcellular localization of multiple copies of genes encoding for enzymes of the OPPP. Such an analysis identified putative cytosolic and plastidic forms of Glc6PDHase, 6PGDHase, Rib5P isomerase, and Rub5P 3-epimerase, whereas TK and TA are predicted only as plastidic enzymes. In other words, Arabidopsis has the genetic capacity to convert cytosolic Rub5P to Rib5P and Xyl5P, but any further rearrangement of the carbon backbone to regenerate fructose-6-P and triose-P only occur within plastids (Kruger and von Schaewen 2003). Similar results were reported by the group of M.J. Emes, when they analyzed the subcellular distribution of OPPP in *Brassica napus* embryos by measuring activity of the different enzymes (Hutchings et al. 2005). Given the observations regarding the cytosolic non-oxidative phase of OPPP, the extent to which it is operative is doubtful (Averill et al. 1998). To further use cytosolic pentose-P by the non-oxidative part of the OPPP, transport of the metabolite across the plastid envelope would be required, and in fact in the inner chloroplast membrane was identified a phosphate translocator protein that preferentially catalyses the counter exchange of Xyl5P, triose-P, and Pi (Eicks et al. 2002).

As in plastids, the key regulatory step in the cytosolic OPPP seems to be that catalyzed by Glc6PDHase although the characterization of the regulatory properties for this cytosolic enzyme is scarce. An early report indicated that both chloroplastidic and cytosolic Glc6PDHases are inactivated in response to light and DTT (Anderson et al. 1974). More recent works pointed out that the two cytosolic isoforms of G6PDHase exhibit differences in modulation, with one being slightly activated by reductants and the other one behaving insensitive to redox signaling (Hauschild and von

Schaewen 2003, Hutchings et al. 2005, Wakao et al. 2008, Wakao and Benning 2005). Current data make difficult to understand the exact role played by cytosolic forms of this enzyme. Arabidopsis single and double null mutants of cytosolic Glc6PDHases indicate the occurrence of alternative, compensatory mechanisms to supply NADPH in the cytosol (Wakao et al. 2008). Since the double mutant showed increased seed oil content and mass, it was concluded that a metabolic change takes place, possibly with increase in the flux of carbon through glycolysis.

In addition to the incomplete OPPP, in the cytosol of plant cells other enzymes were reported as main sources of NADPH. A particular case is non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (np-Ga3PDHase; EC 1.2.1.9) that catalyzes the irreversible oxidation of Ga3P to 3-phosphoglycerate (3-PGA), specifically using NADP<sup>+</sup> as a cofactor, thus generating NADPH according to the reaction (Habenicht 1997, Iglesias 1989):



The np-Ga3PDHase has been identified as a member of the aldehyde dehydrogenase superfamily (Habenicht 1997), and its occurrence is restricted to some specialized eubacteria (Boyd et al. 1995, Brown and Wittenberger 1971, Fourrat et al. 2007, Habenicht 1997, Iddar et al. 2005), archaeobacteria (Brunner et al. 1998), and to the cytosol of green algae and higher plants (Arnon et al. 1954, Bustos and Iglesias 2002, Gomez Casati et al. 2000, Iddar et al. 2002, Iglesias et al. 1987, Iglesias 1989, Iglesias and Losada 1988, Jacob and D'Auzac 1972, Marchal et al. 2001, Mateos and Serrano 1992, Pupillo and Faggiani 1979). The enzyme is coded by a single gene (*gapN*) and exhibits a tetrameric quaternary structure of about 200 kDa (Bustos and Iglesias 2002, Gomez Casati et al. 2000, Habenicht 1997, Iddar et al. 2002). In photosynthetic cells, np-Ga3PDHase is involved in a shuttle system for the export of NADPH photosynthetically generated, from the chloroplast to the cytosol (Kelly and Gibbs 1973). This shuttle system involves the inter-exchange of triose-P and Pi between the chloroplast and the cytosol through the triose-P/Pi translocator of the chloroplast envelope. On the other hand, the enzyme plays a key role in plants accumulating acyclic polyols (such as in celery) by supplying the NADPH necessary for the synthesis of the reduced sugars (Gao and Loescher 2000, Rumpho et al. 1983). In nongreen cells, the enzyme would couple NADPH production necessary for anabolism with glycolysis (Habenicht 1997).

The occurrence of np-Ga3PDHase in the cytosol of plant cells establishes an alternative for glycolysis (see Figure 7.2), where Ga3P can be metabolized to 3-PGA either by the couple of phosphorylating, NAD-dependent Ga3PDHase (EC 1.2.1.12), and 3-PGA kinase (EC 2.7.2.3) or by np-Ga3PDHase (Iglesias 1989, Plaxton 1996). In the first case, NADH and ATP are produced in the pathway, whereas the second route renders NADPH but not ATP (Iglesias 1989). This branch point in glycolysis is expected to be regulated in order to effectively modulate the production of energetic and reductive power within the cell (Plaxton 1996). In fact, it has been shown that, in non-photosynthetic tissues of plant cells, np-Ga3PDHase is a target for posttranslational regulation by phosphorylation (Bustos and Iglesias 2002). The phosphorylated enzyme exhibits distinctive kinetic properties after interaction with 14-3-3 regulatory proteins (Bustos and Iglesias 2003, 2005). Also, recent studies have demonstrated that the enzyme plays a pivotal role in carbon and energy metabolism in plants under both, physiological and oxidative stress conditions (Bustos et al. 2008, Rius et al. 2006). An Arabidopsis mutant lacking np-Ga3PDHase was found to have altered morphology of the siliques, inhibited glycolytic flux, decreased CO<sub>2</sub> fixation capacity, and increased oxidative stress (Rius et al. 2006). Concurrently, the absence of np-Ga3PDHase elicited an induction of Glc6PDHase activity resulting in increased levels of NADPH. These results, taken together with that obtained for the cytosolic Glc6PDHases Arabidopsis mutants support the idea that plants compensate the disruption of one pathway generating NADPH by boosting enzymes involved in alternative routes operating within the cytosol.

Another enzyme considered as NADPH producer in the cytosol of plant cells is the non-photosynthetic isoform of NADP-malic enzyme (NADP-ME; L-malate:NADP oxidoreductase

[oxaloacetate decarboxylating], EC 1.1.1.40). NADP-ME is a widely distributed enzyme involved in different metabolic pathways that catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO<sub>2</sub>, and NADPH in the presence of a divalent cation (Drincovich et al. 2001). Cytosolic isoforms of NADP-ME have been linked to plant-defense responses and to lignin biosynthesis by providing NADPH, as well as to control pH by balancing synthesis and degradation of malate (Smirnov and Wheeler 2000). Plant non-photosynthetic, cytosolic NADP-ME has been characterized with respect to kinetics, regulation, and function (Detarsio et al. 2008, Drincovich et al. 2001, Maurino et al. 1996, 2001, Smirnov and Wheeler 2000). Utilizing genetically transformed Arabidopsis plants over-expressing rice cytosolic NADP-ME was found to have an increase in the cytosolic NADPH/NADP<sup>+</sup> ratio, which confers salt tolerance to transgenic seedlings (Cheng and Long 2007, Liu et al. 2007).

With this background, it can be concluded that NADPH production in the cytosol of plant cells is not strictly dependent on a particular system (Figure 7.2). Rather, it can be visualized as an integrated complex metabolic network in which different primary metabolic pathways interact in order to assure the supply of reducing power demands of the cell and/or to ameliorate oxidative stress situations.

## 7.5 OXIDATIVE SPECIES SCAVENGING SYSTEMS IN PLANT CELLS

### 7.5.1 ANTIOXIDANT MOLECULES AND REDOX COFACTORS

#### 7.5.1.1 Glutathione

Reduced glutathione (GSH) is a tripeptide ( $\gamma$ -glutamyl-cysteinyl-glycine) that exists interchangeably with the oxidized form, GSSG, and is vital for normal cellular function. The GSSG/GSH couple has a standard reduction potential ( $E^{\circ}$ ) of  $-240$  mV, which makes GSH a moderate reducing agent (Banerjee 2008). Reduced GSH pools are maintained by the action of the enzyme glutathione reductase (GR), a pyridine nucleotide disulfide reductase that transfers electrons from NADPH to GSSG to generate GSH (Smirnov 2005). *De novo* synthesis of GSH occurs in two ATP-dependent steps catalyzed by glutamate cysteine ligase and  $\gamma$ -glutamyltranspeptidase. In addition, certain plants contain tripeptide homologues of GSH, in which the carboxy terminal glycine is replaced by other amino acids (Noctor and Foyer 1998). GSH is found primarily in eukaryotes and gram-negative bacteria, although a select number of gram-positive prokaryotes have also been shown to utilize it (Banerjee 2008). In eukaryotic systems, approximately 90% of the intracellular GSH pool resides in the cytoplasm, with the remainder in organelles such as the mitochondria, the endoplasmic reticulum, and the nucleus (Noctor and Foyer 1998). In plant cells, biosynthesis of GSH takes place in the cytosol and the chloroplast (Smirnov 2005).

GSH is the predominant nonprotein thiol in plants and its physiological significance may be divided into two categories: sulfur metabolism and antioxidant defense (Scheibe 1991, Smirnov 2005). GSH is a product of the primary sulfur metabolism acting as a form of transport and storage of reduced sulfur and being important as antioxidant and redox buffer (Banerjee 2008, Buchanan and Balmer 2005). GSH is relevant for phytochelatin synthesis and is thus crucial for detoxification of heavy metals such as cadmium and nickel (Abhilash et al. 2009, Noctor and Foyer 1998). In addition, GSH is substrate of several enzymes (Figure 7.2), such as glutathione-S-transferases (GST) in the detoxification of xenobiotics (Kulinskii and Kolesnichenko 2009, Smirnov 2005), for glutathione peroxidase (GPX) (Dietz et al. 2006), and phospholipids hydroperoxide glutathione peroxidase (PHGPX) (Smirnov 2005) in the reduction of H<sub>2</sub>O<sub>2</sub> and LOOH, respectively. Also, it functions as electron donor for GRXs (Lillig et al. 2008) in the mixed-disulfides reduction of proteins.

#### 7.5.1.2 Ascorbate

Ascorbate (L-threo-hex-2-enono-1,4-lactone, or vitamin C) is well known for its radical-scavenging capacity and is the most abundant small, water-soluble molecule, antioxidant in plants (Smirnov 2005).

In addition to this main role, the moderately positive standard redox potential of the ascorbate/monodehydroascorbate couple ( $E^{\circ'} = 280 \text{ mV}$ ) makes this compound an excellent one-electron donor to a large variety of enzymes, in particular, oxygenases and hydroxylases (Noctor and Foyer 1998). At physiological pH, ascorbic acid (AA;  $\text{p}K_1 = 4.2$  and  $\text{p}K_2 = 11.8$ ) is predominantly present as the ascorbate anion (Banerjee 2008). The latter readily loses an electron from its *ene*-diol group to produce the monodehydroascorbate (MDHA) radical. Further oxidation results in dehydroascorbate (DHA) (Banerjee 2008). The conjugated structure of the five-atom lactone ring containing an *ene*-diol group allows stabilization of the free radical one-electron oxidation product, MDHA, by delocalization of the unpaired electron (Noctor and Foyer 1998). The ability of ascorbate to donate one electron and the relatively low reactivity of the resulting MDHA radical is the basis of its biologically useful antioxidant and free-radical scavenging activity. Paradoxically, ascorbate can also function as a pro-oxidant agent by reducing metals ions such as  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ . Ferrous iron thus formed can then catalyze the generation of the  $\cdot\text{OH}$  from  $\text{H}_2\text{O}_2$  by the Fenton reaction (Banerjee 2008, Halliwell 2006).

All plants and animals, except primates and guinea pigs, can synthesize ascorbic acid (Noctor and Foyer 1998). In plants, ascorbate can accumulate to millimolar concentrations in both photosynthetic and non-photosynthetic tissues. Synthesis occurs from the relatively rare sugar L-galactose via L-galactono-1,4-lactone in the Smirnoff–Wheeler–Running pathway (Linster and Clarke 2008, Smirnoff and Wheeler 2000). This two-step oxidation is catalyzed by the NAD-dependent L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase. L-galactose is provided as GDP-L-galactose, which itself originates through epimerization from GDP-mannose (Noctor and Foyer 1998, Smirnoff and Wheeler 2000). In the ascorbate catabolism, L-galactose is converted to oxalate, tartrate, and threonine. At the present time, the enzymes involved in the cleavage of the ascorbate C-skeleton to produce these organic acids have not been identified (Noctor and Foyer 1998).

In plant cells, the ascorbate recycling is performed by direct reduction of DHA by two molecules of GSH (Figure 7.2). This reaction is thermodynamically feasible and has been demonstrated in cell-free systems (Banerjee 2008, Meyer 2008, Noctor and Foyer 1998). In addition, plants also contain proteins with GSH-dependent DHA reductase activity (Meyer 2008, Smirnoff and Pallanca 1996). In the photosynthetic electron transport chain, electrons are derived from the photooxidation of water by photosystem II and are transferred to MDHA by ferredoxin (Smirnoff 2000). Plants have two enzymes that catalyze ascorbate oxidation: APX (Smirnoff 2005) and ascorbate oxidase (AO) (Dawson et al. 1975). The APX catalyzes the ascorbate-dependent reduction of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  (Shigeoka et al. 2002). APX is central in the antioxidant function of ascorbate in plants, with members found in all subcellular compartments (Dawson et al. 1975). AO, a member of the blue copper oxidase family, is a glycoprotein localized at the apoplastic level (Pignocchi and Foyer 2003), its activity being higher in expanding cucurbit fruits tissues (Arrigoni 1994).

### 7.5.1.3 $\alpha$ -Tocopherol

$\alpha$ -Tocopherol is a potent lipid-soluble antioxidant that is synthesized exclusively by plants and is the most biologically active of the eight forms of vitamin E (Banerjee 2008). This antioxidant is abundant in seeds, linked with triacylglycerols in the oil bodies and probably in glyoxysomes, where they are oxidized during germination. In addition, it is very abundant in thylakoid membranes containing PUFA and are in close proximity to ROS produced during photosynthesis (Zingg 2007). The hydrophobic phytyl side chain gives  $\alpha$ -tocopherol the lipid solubility, which anchors the antioxidant chromanol ring to the membrane (Smirnoff and Wheeler 2000).

The antioxidant capacity of  $\alpha$ -tocopherol is a result of its capacity to quench free radicals by becoming a stable radical itself (Zingg 2007). The chromanol ring can donate a single electron resulting in generation of a resonance-stabilized tocopheroxyl (tocopheryl or chromanoxyl) radical. This mechanism is the basis of its ability to prevent lipid peroxidation chain reactions. The reaction between lipid peroxy radicals and tocopherol originates hydroperoxides that may be reduced by PHGPX (Shao et al. 2008). The tocopheroxyl radical can be reduced to restore  $\alpha$ -tocopherol by



other antioxidants, such as ubiquinol, ascorbic acid, and, indirectly, dihydrolipoic acid (Banerjee 2008). On the other hand, a putative non-antioxidant function has been ascribed to  $\alpha$ -tocopherol as stabilizing cellular membrane structure by interacting with polyunsaturated fatty acyl chains (Smirnov 2005).

#### 7.5.1.4 Carotenoids

Carotenoids are the most abundant pigmented plant-derived compounds, which impart the red and yellow color to fruits and vegetables (Lu and Li 2008). These compounds are isoprenoids, consisting primarily of eight joined isoprene units with a rigid backbone due to the presence of 3 to 15 conjugated double bonds, which confer antioxidant properties. Some chemical modifications include cyclization of the carbon skeleton at one or both ends (Banerjee 2008, Britton 1989). In plants, carotenoids are essential to survival; they directly participate in photosynthesis and reactions to limit vast amounts of ROS produced in chloroplasts (Lu and Li 2008). Oxygen-containing carotenoids (xanthophylls) are integral components of photosystem II (PSII) and the light-harvesting complex (Banerjee 2008). Zeaxanthin is implicated in non-photochemical quenching of excitation energy in PSII, in which excess of energy in PSII is transferred to zeaxanthin and reradiated as heat (Ivanov et al. 2008). In addition, several carotenoids participate in defense against photoinduced damage (Smirnov 2005). Both  $\alpha$ -tocopherol and ascorbic acid can reduce carotenoid radicals, which might ascribe for the observed synergistic interactions between these three important antioxidants (Banerjee 2008, Smirnov 2005).

#### 7.5.1.5 Flavonoids

Flavonoids are a group of polyphenolic compounds produced as secondary metabolites by plants (Banerjee 2008). The general structure of plant-derived flavonoids presents two aromatic benzene rings linked through three carbons that can form an oxygenated heterocycle (Smirnov 2005). A major integrant of the group is quercetin, which is the most abundant flavonoid of this class. Other examples are isoflavones (phytoestrogens), found in high abundance in legumes (Veitch 2009). Citrus contain hesperidin (a glycoside of the flavanone hesperetin), quercitrin, rutin (two glycosides of the flavonol quercetin), and the flavone tangeritin (Gattuso et al. 2007). Main flavonoids in green tea are kaempferol and catechins (catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate) (Butt and Sultan 2009). Grape skins contain important amounts of flavonoids as well as other polyphenols. Both red and white wine contain flavonoids; nevertheless, since red wine is produced by fermentation in the presence of the grape skins, it contains higher amounts of flavonoids and other polyphenolics such as resveratrol (Dohadwala and Vita 2009). Chelation of redox-active metals is the principal defensive antioxidant activity of flavonoids. Thus, they prevent peroxy radical and lipid peroxidation, scavenging of hydroxyl and peroxy radicals, and quenching of superoxide radicals and singlet oxygen (Banerjee 2008).

#### 7.5.1.6 NAD(P)<sup>+</sup>

The coenzyme pyridine nucleotide, nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and its phosphorylated derivative (NADP<sup>+</sup>) are the universal energy carriers performing reversible two-electron (specifically one hydride) transfer in a variety of essential metabolic reactions in all living organisms (Pollak et al. 2007). The coenzyme NAD<sup>+</sup> (oxidized form) participates mainly in oxidative reactions by accepting electrons from energy-rich substrates (e.g., in glycolysis). On the other hand, NADPH (reduced form) acts principally as electron donor in reductive biosynthetic reactions and has an important role in oxidant production and antioxidant defense in the plant cells by means of NADPH-dependent enzyme, such as NADPH oxidase and GR, respectively (Banerjee 2008). The  $E^{\circ'}$  for the two-electron reduction of the NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH couples are similar (about -320 mV) (Schafer and Buettner 2001). The fact that NAD<sup>+</sup> also participates in other reactions that are not related with redox metabolism is worth mentioning. Such are the cases when NAD<sup>+</sup> is involved in protein modification processes such as poly-ADP-ribosylation of transcription



factors in the nucleus (Ziegler 2000), or in signaling pathways as a precursor of the second messenger cyclic-ADP-ribose (Guse 2004), as well as when it acts as a substrate of a group of enzymes called sirtuins (Blander and Guarente 2004). The latter use  $\text{NAD}^+$  to remove acetyl groups from histones, being implicated in the regulation of transcription, apoptosis, and stress resistance (Trapp and Jung 2006).

Biosynthesis of  $\text{NAD}^+$  in plants occurs through two metabolic pathways. It is produced either in a *de novo* pathway from tryptophan or aspartic acid, or in salvage pathways by recycling pre-formed components such as nicotinamide (Banerjee 2008, Katoh et al. 2006).  $\text{NADP}^+$  is generated by phosphorylation of hydroxyl group in the 2' position of the ribose ring that carries the adenine moiety (Banerjee 2008). In chloroplasts,  $\text{NADP}^+$  is reduced by ferredoxin- $\text{NADP}^+$  reductase in the last step of the electron chain of the light reactions of photosynthesis (Smirnov 2005). The  $\text{NADPH}$  produced is then used as reducing power for carbohydrate biosynthesis in the Benson–Calvin cycle (Smirnov 2005). The balance between the oxidized and reduced forms of nicotinamide adenine dinucleotide is called the  $\text{NAD(P)}^+/\text{NAD(P)H}$  ratio. This ratio is an important component of what is called the redox state of a cell, a measurement that reflects both the metabolic activities and the health of cells (Schafer and Buettner 2001).

### 7.5.1.7 Flavin

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) participate in a variety of enzyme-catalyzed reactions as non-covalently or covalently bound redox cofactors. A characteristic property of flavins is their ability to couple one- and two-electron transfer reactions between substrates and different electron carriers. In consequence, flavins can equilibrate the reaction between quinone (oxidized), semiquinone (one-electron reduced), and hydroquinone (two-electron reduced) species with reversible electron transfer occurring across the isoalloxazine ring of the flavin. The  $E^{\circ'}$  of free FAD is  $-219\text{ mV}$  in solution (Banerjee 2008) whereas enzyme-bound flavin has an  $E^{\circ'}$  between  $+100$  to  $-400\text{ mV}$  (Schafer and Buettner 2001). The latter contributes to the fact that many oxidoreductases, called flavoenzymes or flavoproteins, are involved in a significant diversity of reactions, such as dehydrogenation, electron transfer, dehalogenation, hydroxylation, luminescence, DNA repair, and disulfide reduction (Banerjee 2008, Rouhier et al. 2002, Smirnov 2005).

The reactivity of enzyme-bound reduced flavin with  $\text{O}_2$  is influenced by the active site environment and substrate/product complexation (Miura 2001). Reaction of reduced flavin with  $\text{O}_2$  proceeds initially through a one-electron reduction of oxygen to form a flavin semiquinone and  $\text{O}_2^{\cdot-}$  pair. Subsequent events then lead to the formation of  $\text{H}_2\text{O}_2$  (Miura 2001). The ability of reduced flavins to carry out the one-electron reduction of  $\text{O}_2$  to  $\text{O}_2^{\cdot-}$  implies to flavins with intracellular oxidative stress and pathogen defense (Yoshioka et al. 2009). In addition, some flavoenzymes are involved in regulatory and signaling pathways. An example is the apoptotic inducing factor, which is a mitochondrial flavoenzyme that shows  $\text{NADH}$  oxidase and DNA binding activities (Fleury et al. 2002, Le Bras et al. 2005). Another interesting class of flavoenzymes is the structurally related cryptochromes and DNA photolyases that are activated by blue light (Muller and Carell 2009). Cryptochromes are implicated in photosensitive signaling pathways that set the circadian clock and regulate plant growth and development. Phototropins are other group of light-sensible flavoproteins that contribute to adaptive responses to blue light in plants, thus modulating a wide variety of physiological events such as plant development, seed germination, and phototropism. (Demarsy and Fankhauser 2009, Takagi 2003).

## 7.5.2 ANTIOXIDANT ENZYMES

### 7.5.2.1 Thioredoxin System

TRXs are small redox proteins (usually around 12 kDa) with two cysteine residues in its active site within the conserved WCG/PPC motif. TRX can exist either in reduced (dithiol) or in oxidized (disulfide) form (Banerjee 2008). The function of TRX as a thiol reductant is based on the fast

reaction between reduced TRX and disulfide substrates that is linked to the reductive power of NADPH and ferredoxin systems, respectively, to disulfide reduction of target proteins (Smirnov 2005). The redox potential of TRXs is critical for activity, with the value found for plant TRXs ranging between  $-285$  and  $-350$  mV (Buchanan and Balmer 2005). Plants have an unusually high number of TRX encoding genes implicated in photosynthetic regulation. The *A. thaliana* genome encodes at least 22 TRX isoforms, which are classified in six different groups: f, m, x, and y in chloroplasts, o in mitochondria, and h in several cell compartments (cytosol, mitochondria, endoplasmic reticulum) as well as outside the cell (Gelhaie et al. 2005, Montrichard et al. 2009). TRX may control the activity of enzymes, receptors, and transcription factors via its protein disulfide reductase activity; for example, photosynthetic enzymes are controlled by specific TRXs via light. TRX<sub>m</sub> and TRX<sub>f</sub> are specific for target enzymes like malate dehydrogenase and fructose biphosphatase, respectively (Smirnov 2005).

TRXs were identified as the first thiol–disulfide exchange proteins in plants (Gelhaie et al. 2005). They have a common tertiary protein structure, the so-called thioredoxin fold. The same domain structure is found in many other thiol-proteins such as the GRX, GST, GPX, PRX, and protein disulfide isomerase (Martin 1995). Reduced TRX acts to directly reduce protein disulfides by means of fast thiol–disulfide interchange reactions (Bindoli et al. 2008). Initially, reduced TRX non-covalently docks to a target protein through a hydrophobic interaction surface and hydrogen bonds between residues of the TRX backbone and the target protein. This is followed by nucleophilic attack of the thiolate of the N-termini cysteine (comprised in the CXXC active domain) on the target disulfide performing a thiol–disulfide exchange reaction and rendering a transient protein–protein complex disulfide intermediate. Then, the intramolecular attack of C-termini cysteine cleaves the disulfide forming oxidized TRX and the reduced target protein (Banerjee 2008, Buchanan and Balmer 2005).

As illustrated by Figure 7.2, in plants, TRXs are components of two redox systems found in different cell compartments (Smirnov 2005). The ferredoxin system is located in chloroplasts and involves ferredoxin (Fd, an iron–sulfur protein), ferredoxin–thioredoxin reductase (FTR) and TRX f, m, x, and y (Gelhaie et al. 2005, Schurmann and Jacquot 2000). In this system, electrons flow via thiol–disulfide exchange intermediates from Fd, reduced in the light (via photosystem I), to the target protein according to the sequence: light  $\rightarrow$  Fd  $\rightarrow$  FTR  $\rightarrow$  TRX  $\rightarrow$  target protein (Buchanan and Balmer 2005, Scheibe 1991). The system turns into oxidized form in the dark in the presence of O<sub>2</sub> (or oxidized TRX, GSSG, or ROS) (Montrichard et al. 2009). The chloroplast consequently differs from the cytoplasm in undergoing changes from a reductive state in the light to a more oxidative one in the dark (Buchanan and Balmer 2005). The FTR is the central enzyme of the ferredoxin system that transfers a redox signal received from Fd to TRX by a unique mechanism involving a 4Fe-4S cluster and a disulfide bridge, thereby transforming the signal from an “electron signal” to a “thiol signal” (Buchanan et al. 2002, Schurmann 2003).

The NADPH-dependent TRX system is localized both, in the cytosol and mitochondria (Gelhaie et al. 2005). The system includes an FAD-containing enzyme, an NADPH-dependent thioredoxin reductase (NTR, a low molecular weight thioredoxin reductase) that in *A. thaliana* exists as two identical forms (A and B, coded by two distinct genes) (Meyer et al. 2005, 2008). The two NTR have been localized in either the cytoplasm or mitochondria (Meyer et al. 2005). The enzymes transfer electrons from NADPH to TRX<sub>h</sub> (in the cytoplasm) or TRX<sub>h</sub> and TRX<sub>o</sub> in mitochondria (follow a thiol/disulfide exchange mechanism), according to the sequence: NADPH  $\rightarrow$  NTR  $\rightarrow$  TRX  $\rightarrow$  target protein (Buchanan and Balmer 2005, Gelhaie et al. 2005, Smirnov 2005). In addition, recent studies found the existence, in chloroplasts, of a modified type of NTR called NTRC. The latter presents a TRX domain in the C-terminal extension (Kirchsteiger, Pulido, Gonzalez et al. 2009). This form of NTR operates as a complete NADPH-dependent TRX system, being able to transfer reducing equivalents from NADPH to BAS1 (a plastidic 2CysPRX) (Alkhalfioui et al. 2007, Moon et al. 2006).

The number of potential functions of the TRX system cannot be detailed here and remain to be confirmed in several cases. Apart from its documented dark/light regulation, the TRX system

participates in responses against environmental stresses (Gelhaye, Rouhier, Navrot et al. 2005). The TRX system is involved in ROS detoxication at least through several PRX isoforms, GPX and MetSO reduction by means of MSRA/B proteins (Montrichard et al. 2009) (see [Figure 7.2](#)).

### 7.5.2.2 Glutathione-Dependent System

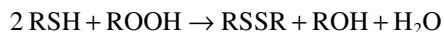
GSH is a key low molecular weight component involved in the protection of plant cells to oxidative stress (Smirnov 2005). As shown in [Figure 7.2](#), in this process, GSH is converted to its oxidized product, GSSG (Noctor and Foyer 1998). A specific flavoenzyme belonging to the disulfide oxidoreductase family, the GR reduce GSSG to GSH using NADPH as electron donor (Rouhier et al. 2008). Most of GRs are homodimeric enzymes with the active site being arranged by both subunits (Rybus-Kalinowska et al. 2009). Each monomer can be divided into three domains: the FAD, the NADP<sup>+</sup>, and the interface domain. GSSG binding site is a bridge between the interface domain of one subunit and the FAD domain of the adjacent subunit (Banerjee 2008, Rybus-Kalinowska et al. 2009).

Alternatively, GSSG can react with protein thiols to form protein–glutathione mixed disulfides (PSSG) in a process called glutathionylation or protein thiolation (Dalle-Donne et al. 2007, Rouhier et al. 2008). Glutaredoxins (GRXs or thioltransferases) are GSH-dependent enzymes responsible for the protein dethiolation reaction (Dalle-Donne et al. 2007). Glutathionylation can either inactivate or activate several enzymes (Buchanan and Balmer 2005), with this regulated and reversible process serving to modulate certain metabolic pathways and in cell signaling (Buchanan and Balmer 2005). The glutathione system (formed by NADPH, GR, GSH and GRX) often functions in parallel with the TRX system in regulating redox homeostasis in the cell.

GRX is a member of the thiol–disulfide oxidoreductase enzyme family and an important component of the GSH system (Lillig et al. 2008). It is a small protein of 10 to 24 kDa that catalyzes the reduction of proteins that are thiolated by GSH (PSSG) (Meyer et al. 2008). The reduction of PSSG is carried out by GSH, which is oxidized to GSSG and recycled to GSH via the recycling system of NADPH and GR. Thus, the electron transfer path is as follows: NADPH → GR → GSH → GRX (Smirnov 2005). It has been established that GRX can reduce protein disulfides (PSSP) using a dithiol mechanism. It can also reduce PSSG, or low molecular weight dithiols (GSSR) via either a monothiol or dithiol mechanism (Lillig et al. 2008). Approximately 30 GRX isoforms were found in *A. thaliana* (Meyer et al. 2008). According to their redox-active center, GRXs are included in three main classes: the CPYC, CGFS, and CCX[C/S] type (Rouhier et al. 2004). CC-type GRXs are only found in higher plants (Meyer et al. 2008). The redox potential of GRXs ranges between –190 and –230 mV, with these proteins performing as electron carriers in the glutathione-dependent synthesis of deoxyribonucleotides by ribonucleotide reductase (Lillig et al. 2008). In addition, GRXs participate in antioxidant defense, performing the DHA reduction (Holmgren and Aslund 1995) and serving as electron donor for several PRXs (Meyer et al. 2008) and for MSRB (Tarrago et al. 2009). Besides their function in antioxidant defense, GRXs were shown to bind iron–sulfur clusters and to deliver the cluster to enzymes on demand (Rouhier et al. 2008). Moreover, in *Arabidopsis*, GRXs are involved in flower development and salicylic acid signaling (Meyer et al. 2008, Rouhier et al. 2008).

### 7.5.2.3 Peroxiredoxin

PRXs are described as a family of thiol-based antioxidant enzymes, ubiquitously found in nature. They catalyze the reduction of H<sub>2</sub>O<sub>2</sub>, a wide variety of organic hydroperoxides (from *tert*-butyl hydroperoxide, cumene hydroperoxide, fatty acid peroxides to complex phosphatidyl choline peroxides), and ONOO<sup>–</sup> at expenses of thiol substrates, according to the equation (Banerjee 2008):



PRXs belong to the group of proteins having the thioredoxin fold, presenting the active site Cys in a TPXC motif (Dietz 2003). Furthermore, important new information on PRXs indicates that they are

not only involved in detoxification of peroxides (Figure 7.2), but also in plant-specific functions. Examples of the latter are the involvement of PRXs in the context of photosynthesis, phloem metabolism, environmental stress, pathogen resistance, proliferation, differentiation, and apoptotic pathways through both known and unknown mechanisms (Dietz 2003, Smirnov 2005).

Mechanistically, PRXs can be classified in three classes: typical 2-CysPRX, atypical 2-CysPRX, and 1-CysPRX (Poole 2007, Rouhier and Jacquot 2002). These enzymes present the same basic catalytic mechanism, in which a redox-active cysteine (the peroxidatic cysteine or R-S<sub>P</sub>H) in the active site is oxidized to a sulfenic acid (R-S<sub>P</sub>OH) by the oxidant substrate. The regeneration process of the oxidized cysteine differentiates three mechanistic classes. The typical and atypical 2-Cys PRX present a second reactive cysteine, named resolving cysteine (R-S<sub>R</sub>H), involved in generating an intersubunit disulfide bond with the sulfenic acid form of the peroxidatic cysteine (to generate R-S<sub>P</sub>-S<sub>R</sub>-R) previous to reduction. This mechanism is followed by the typical 2-CysPRX with the R-S<sub>R</sub>H on a partner subunit (Dietz et al. 2006). On the other hand, the atypical 2-CysPRX performs the same chemical mechanism (disulfide bond formation) for catalysis except that both, the R-S<sub>R</sub>H and the R-S<sub>P</sub>H, pertain to the same monomer, thus forming an intrasubunit disulfide bond previous to reduction (Smirnov 2005). The third type of PRXs, the 1-CysPRX, bypasses the disulfide bond formation prior to reduction (Banerjee 2008). The mechanism for recycling 2-CysPRX involves TRX, GRX, or another CXXC-containing redox module protein (Dietz 2003) such as CDSP32 (Rey et al. 2005), NTRC (Kirchsteiger et al. 2009), and cyclophilin (Dietz 2007). The ways for 1-Cys PRX reduction are quite uncertain, although several studies demonstrated that both, ascorbate and GSH, perform as reducing substrates (Dietz 2003). The redox potential of plastidic PRXs ranges between -307 to -325 mV (Dietz et al. 2006).

The *A. thaliana* genome contains 10 *prx* genes belonging to four different groups: 1-CysPRX, typical 2-CysPRX, PRXQ (atypical 2CysPRX), and type II PRX (atypical 2CysPRX) (Smirnov 2005). Some of the PRX transcripts have been detected in all analyzed plant tissues though the expression of each individual member of the *prx* gene family may differ significantly. The accumulation of transcripts encoding chloroplastic PRX shows a correlation with chlorophyll both in tissue distribution and during leaf development (Dietz 2003).

Even when numerous studies indicate that PRXs exclusively act as antioxidants by reducing peroxides, recent works have begun to uncover alternative roles played by these proteins (Rouhier and Jacquot 2002). Day after day evidences increase respect that H<sub>2</sub>O<sub>2</sub> is utilized as a signaling molecule to regulate a variety of important cellular functions (Neill et al. 2002, Noctor 2006, Vranova et al. 2002). As abundant and ubiquitous peroxidases, the peroxidase activity of PRXs is central in the regulation of these functions. Recent studies suggest that regulation of the peroxidase and chaperone activities of these multifunctional enzymes is an important feature for the H<sub>2</sub>O<sub>2</sub>-mediated signal transduction in plants (Hall et al. 2009). Moreover, the activity and other properties of some PRXs (particularly the typical 2-CysPRX) are regulated by over-oxidation (Banerjee 2008), nitrosylation (Romero-Puertas et al. 2007), and/or phosphorylation (Aran et al. 2009). Therefore, PRXs are probably playing a key role in modulating ROS-RNS regulatory networks as well as the intensity in the response.

Numerous evidences support that PRXs modulate plant cells signaling by several mechanisms (Dietz et al. 2006). For example, it was described that PRXs are involved in (i) modulating H<sub>2</sub>O<sub>2</sub> concentration [H<sub>2</sub>O<sub>2</sub> is a major ROS signal during adaptation and development by modifying the nuclear gene expression (Foyer and Noctor 2005)], (ii) altering lipid hydroperoxide levels [PRX can change levels of lipid peroxide-derived oxylipins that, in turn, also modulate plant responses at the level of gene expression (Mueller and Berger 2009)], (iii) detoxifying ONOO<sup>-</sup> [which mediates nitration of proteins (Aran et al. 2009)], (iv) "spending" electron donors [thus affecting redox state of other targets], and (v) regulating the redox state of interacting thiol proteins [Prx indirectly influence the redox state and possibly activity of other targets that are regulated by redox interactions with these donors]. PRXs may be considered as peroxide sensors [transmitting the information of high peroxide concentrations to other thiol proteins (Dietz 2003)], and they can

also undergo conformational changes and interact with non-thiol proteins [2CysPRX may act as molecular chaperones under oxidative conditions, interacting to non-thiol proteins and other cell structures (Aran et al. 2009, Hall et al. 2009)].

#### 7.5.2.4 Superoxide Dismutase

SODs are metalloproteins that catalyze dismutation of  $O_2^{\cdot-}$  into  $O_2$  and  $H_2O_2$ , thus representing a relevant antioxidant defense in photosynthetic cells (Smirnov 2005). *In vivo*, SOD constitutes the first line of defense against ROS, protecting cells and tissues from oxidative destruction (Hancock 1997). Plant cells have several forms of SOD, which mainly vary on the metal cofactor present: copper and zinc (CuZnSOD), manganese (MnSOD), or iron (FeSOD). CuZnSODs are generally homodimeric enzymes with a subunit molecular mass of ~16 kDa, containing one  $Cu^{2+}$  and one  $Zn^{2+}$  per subunit (Tainer et al. 1983). CuZnSODs are found in the cytosol as well as in many of the organelles of eukaryotic cells (Hart et al. 1999).  $Cu^{2+}$  constitutes the catalytic center, while  $Zn^{2+}$  provides a structural helping role (Hart et al. 1999). MnSODs localize in the mitochondrial matrix of animals and plants (Jackson et al. 1978). Mitochondrial MnSOD is a homotetrameric protein with a subunit molecular mass of 23 kDa (Jackson et al. 1978). FeSODs are constitutive enzymes that can be found in plastids (Bridges and Salin 1981).

In biological systems,  $O_2^{\cdot-}$  can undergo dismutation (velocity  $\sim 10^5 \text{ m}^{-1}\text{s}^{-1}$  at pH 7.0) (Banerjee 2008). The enzyme SOD is biologically essential because  $O_2^{\cdot-}$  reacts even faster with another biological radicals such as the NO, generating a most toxic specie, the ONOO<sup>-</sup> (Valderrama et al. 2007). SOD has the fastest turnover number of any known enzyme ( $\sim 10^9 \text{ m}^{-1}\text{s}^{-1}$ ), being this reaction only limited by the collision frequency between the enzyme and the substrate  $O_2^{\cdot-}$  (Banerjee 2008).

#### 7.5.2.5 Glutathione S-Transferase

GSTs catalyze the nucleophilic attack of GSH upon an electrophilic substrate (Smirnov 2005). GSTs participate in xenobiotic detoxification (Figure 7.2); however, they also catalyze specific reactions in a number of biosynthetic and catabolic pathways and play an important role in defense against oxidative stress by reducing ROOH and DHA (Banerjee 2008). In addition, it has been clearly established that many GSTs present GPX activity; whereas is less clear if they are involved in other reactions related with stress signaling and protein binding (Noctor and Foyer 1998). In plants, all GSTs so far described form homodimers of 50 kDa, being classified in four major groups: phi-, tau-, zeta- and theta-GST (Banerjee 2008). Recently, two additional groups were identified in *A. thaliana* (Dixon et al. 2002). The fifth group, omega-GST, is formed by four putative small DHAR with an active site represented by the CPFC/S motif (Dixon et al. 2002, Smirnov 2005). DHAR regenerates ascorbate from DHA using GSH as electron donor (Figure 7.2). The sixth subgroup, lambda-GST, comprise two members that exhibit no DHAR activity but are active as GSH-dependent thiol transferases, suggesting that they might function in dethiolation of S-glutathionylated proteins accumulated under oxidative stress situations (Dixon et al. 2002, Rouhier et al. 2008).

#### 7.5.2.6 Ascorbate Peroxidase

APX is a hemoprotein that, in contrast to catalase and GPX, appears to be a unique enzyme found mainly in plants, algae, and some protozoa (Noctor and Foyer 1998, Wilkinson et al. 2002). The enzyme participates in detoxification of  $H_2O_2$  (Figure 7.2) using ascorbate as reducing substrate, according to reaction:



APX is present in almost every compartment of the plant cell and it participates in the detoxification of  $H_2O_2$  as part of the ascorbate–glutathione or Asada–Halliwell–Foyer pathway (Hiner et al. 2002). Initially, APX activity was detected in intact chloroplasts and algae (Kelly and Latzko 1979,

Shigeoka et al. 1980). Three forms of chloroplastidic APX were reported: thylakoid-APX, stromal-APX, and soluble-APX (localized in the lumen) (Smirnoff 2000). In *Arabidopsis*, nine different genes encode APX enzymes, four of them encoding chloroplastic isozymes (Kubo et al. 1992). Two stromal APXs are dually targeted to the stroma and the mitochondrial intermembrane space (Smirnoff 2000). In addition, two different APXs are thought to be targeted to peroxisomes and glyoxysomes (Hoshi and Heinemann 2001, Nito et al. 2001) and two are localized in cytoplasm (Santos et al. 1996). Finally, there are two microsomal APXs that might bind to the external surface of glyoxysomes or be transported into peroxisomes (Lisenbee et al. 2003).

The function of APX is dependent of the availability of ascorbate and, in some cases, GSH (Smirnoff 2005). Cellular pools of these antioxidants are maintained in reduced form by enzymatic systems such as GR, DHAR, GRX, and GST (Buchanan and Balmer 2005). In addition, the enzyme presents higher affinity for  $H_2O_2$  than for organic peroxides, making it a suitable candidate to participate in the removal of the former for signaling purposes (Apel and Hirt 2004, Reddy et al. 2009). Studies with transgenic plants have demonstrated that APX is an important defense enzyme implicated in the elimination of  $H_2O_2$  (Smirnoff 2005). APX might function as component of the  $H_2O_2$ -regulation network of plants, controlling levels of the peroxide used for cellular signaling (Pauly et al. 2006). Thus, in concert with other  $H_2O_2$ -regulation enzymes of the cell, APX balances different cellular systems that generate  $H_2O_2$  in plants (e.g., NADPH oxidase in pathogen response), and also controls levels of  $H_2O_2$  used for signaling during biotic or abiotic stress (Apel and Hirt 2004, Kotchoni and Gachomo 2006).

### 7.5.3 CROSS TALK BETWEEN ANTIOXIDANT SYSTEMS

As it was described, plants have developed efficient enzymatic systems to resist/modulate damage generated by ROS and RNS. The redox cellular status is a crucial mediator for different metabolic processes acting in signaling and regulation of several metabolic and cellular processes; for example, the regulation of enzymatic activities, gene expression, growth differentiation, pathogen resistance, and apoptosis. Main pathways involved in the maintenance of the intracellular redox homeostasis are the TRX- and the GSH-dependent systems. These systems do not act in an individual and isolated manner, but they interact with other enzymatic and nonenzymatic components to prevent, repair, and regulate oxidative damages. In this way, we propose the scheme depicted in Figure 7.2 for reductive/oxidative equivalents flux between the main plant redox components described above. As the picture shows, different antioxidant and repair systems interact to exert specific functions related with redox homeostasis inside the cell. In this scenario, it is worth highlighting the position of the NADPH as a central donor supplying reducing equivalents for all the different reducing systems.

## 7.6 OXIDATIVE DAMAGE REPAIR SYSTEMS OPERATING IN PLANTS

All reactive species can modify different macromolecules to generate a cellular damage situation (Moskovitz 2005). For this reason, cells have many protective systems helping to eliminate or minimize injurious molecular species or to repair the oxidative damage caused on cellular components (Banerjee 2008). Particularly, oxidation of proteins can generate conformational changes and, in some cases, loss of function (Friguet 2006). The amino acids most susceptible to the oxidation are histidine, tryptophan, tyrosine, cysteine, and methionine; the latter two being the most sensitive (Moller et al. 2007). Systems for protein repair found in plants include SRXs and methionine sulfoxide reductases (Smirnoff 2005).

### 7.6.1 SULFIREDOXINS

As discussed in Section 7.5.2, typical 2CysPRX in eukaryotes are susceptible of hyperoxidation by excess of oxidizing substrate (Kiley and Storz 2004). As a result of this, the  $R-S_pOH$  (formed

during the catalytic cycle) generates the  $R-S_pO_2H$  (a more oxidized sulfur state by two electrons) (Rouhier and Jacquot 2002). The capacity of this hyperoxidation pathway in regulating PRX activity could provide cells the ability to maintain generation of local of  $H_2O_2$  under controlled levels. Consequently, the system operates maintaining concentrations of the peroxide in ranges compatible with its role in receptor-mediated, redox-dependent signaling processes (Rouhier et al. 2004). Another theory conjectures that PRX hyperoxidation leads to production of a high molecular mass PRX multimeric protein that exhibits chaperone activity to assist in cell recovery from oxidative stress (Aran et al. 2009, Hall et al. 2009). In plants, 2CysPRXs constitute the most abundant PRXs and are principally located in chloroplasts (Dietz et al. 2006). Lower oxidation states of cysteine (e.g., disulfides) are readily reversible; but higher oxidation states (such as  $R-S_pO_2H$ ) were once considered irreversible in a biological scenario (Moller et al. 2007). This point of view was revisited with the discovery of SRX, an enzyme that can reduce  $R-S_pO_2H$  back to  $R-S_pOH$ , in an ATP-dependent manner (Biteau et al. 2003, Chang et al. 2004).

Plant SRX proteins present high identity with orthologs from yeasts and humans, as they have the conserved signature sequence and residues essential for catalysis (Liu et al. 2006). Nevertheless, SRXs from plants possess a distinctive transit peptide directing them to the chloroplast. A study performed with SRX-GFP fusion protein indicated that this protein was targeted to the chloroplast in *Arabidopsis* mesophyll protoplast (Rey et al. 2007). Several experiments revealed that the expression of SRX genes occurred in both vegetative and reproductive organs and the highest transcript level was detected in leaves (Banerjee 2008). In addition, SRX transcript level is considerably augmented under oxidative stress, in parallel with enhanced transcription of 2CysPRX, which is essential in maintaining the chloroplast redox balance (Dietz et al. 2006, Rey et al. 2007).

SRX reduces hyperoxidized PRX in the presence of  $Mg^{2+}$  and ATP, transferring the phosphoryl group from the nucleotide to the PRX sulfinic acid. This generates a sulfinic phosphoryl ester that is the first step required for the activation of this species (Jonsson et al. 2009). The catalytic mechanism includes the nucleophilic attack on the sulfinic phosphoryl ester by the thiolate of the essential cysteine in SRX, thus producing the thiosulfinate linkage between the two proteins (Jonsson et al. 2008). Subsequently, the breakdown of the complex by reaction with another attacking thiolate generates a disulfide bridge in SRX (Roussel et al. 2008). It appears that TRX, and possibly GSH, can serve as reductants in the repairing process (Park et al. 2009, Roussel et al. 2009). On the other hand, numerous investigations show that SRX has differential reactivity toward diverse hyperoxidized PRX targets (Jonsson and Lowther 2007).

### 7.6.2 METHIONINE SULFOXIDE REDUCTASES

We analyzed before (see Section 7.3.2) a general view on the oxidation of sulfur-containing amino acids in proteins and the role of repairing enzymes such as MSRs. Plants have multiple forms of MSRA and MSRB, which are targeted to different cellular compartments (Tarrago et al. 2009). Multiple locations of MSRA and MSRB enzymes indicate that reduction of oxidized methionine residues in proteins takes place independently within the different subcellular locations (Rouhier et al. 2006, 2007). In addition, the overexpression of MSRB2 in *A. thaliana* transgenic plants generates enhanced tolerance to cellular oxidative damage during long nights (Bechtold et al. 2004, Romero et al. 2004).

Several studies indicate that MSRA and MSRB share a similar reaction mechanism (Boschi-Muller et al. 2008, Kauffmann et al. 2005), which proceeds in a series of steps. First, the catalytic cysteine thiolate (situated in the N-terminal part of MSRA and the C-terminal portion of MSRB) attacks the sulfoxide, releasing methionine and producing a sulfenic acid intermediate on the cysteine. Second, the sulfenic acid intermediate is attacked by the resolving cysteine generating an intramolecular disulfide bond. Finally, TRX or other electron donors, such as GRX or GSH, reduce the intramolecular disulfide, regenerating MSR (Tarrago et al. 2009). Most MSRB also contain a

single zinc atom that supports a structural role in these proteins (Kauffmann et al. 2005). In addition, some MSRB lack any resolving cysteines, suggesting that the sulfenic acid intermediate may be directly reduced by TRX or GSH (linking to GRX) (Vieira Dos Santos et al. 2007) or CDSP32 (Rey et al. 2005).

Recent works provided evidence for the existence of a mechanism by which plants perceive  $H_2O_2$  associated to methionine oxidation (Emes 2009). The data indicate that the chemical oxidation of methionine residues by  $H_2O_2$  at key hydrophobic positions within canonical phosphorylation motifs in numerous enzymes inhibits protein kinases binding. This inhibition of kinases (including the calcium-dependent protein kinase and AMP-activated protein kinase families) can be reversed by MSR *in vivo* (Hardin et al. 2009). The authors demonstrate that this mechanism is directly linked to oxidative signals by means of changes in protein phosphorylation, contributing to knowledge on perception of redox signaling in plants.

## 7.7 CONCLUDING REMARKS

Plants, as all aerobic organisms, take advantage of the redox potential of oxygen using it in metabolic pathways. Antioxidants and ROS/RNS/RSS are important interacting systems with different functions in higher plants, which ensure high redox flexibility to the organism. Antioxidants are not passive bystanders in this cross talk, instead they function as key signaling components that constitute a dynamic metabolic interface between cell stress perception and physiological responses (Scandalios 2005). Normal metabolic conditions produce ROS and RNS as by-products. To avoid undesirable effects by these species, plants have effective defense mechanisms, including antioxidant enzymes and free-radical scavengers. Under certain situations, like biotic or abiotic stress as well as the course of development, an induction of ROS and RNS synthesis takes place, that act as signaling molecules, incoming complex downstream effects on both primary and secondary metabolisms. Circumstances like this could result in different situations, the cell responds to the ROS/RNS signal making appropriate adjustments and returning to physiological conditions. Otherwise, ROS/RNS production exceeds the capacity of antioxidant systems, cells are not able to return to homeostasis, oxidative damage is overcome, and genetically programmed cell suicide events are triggered.

It is now well established that most cells can adapt to oxidative stress by altering global gene-expression patterns, including transcription and translation of genes encoding antioxidants as well as other metabolic enzymes, and/or by posttranslational changes operated on proteins that could modify key regulators of redox responses (Grant 2008). In plants,  $H_2O_2$  has been recognized as a second messenger for signals triggered by ROS, and advances have been gained in the understanding of metabolic changes operated in consequence. Also, the role of  $\cdot NO$  and the problem of RNS has been clearly evidenced. However, areas related with regulation of gene expression, modulation of enzymes activity, and coordination/redirectioning of metabolic fluxes require intensive research to reach a complete and comprehensive view of how higher plants maintain levels of oxidative species under control. The use of post-genomics tools, including proteomics and metabolomics, are becoming to be utilized in this field and are critical for advancements in integrative outlooks. The challenge is complex, but the finding of responses to the different open questions foresees relevant consequences. The latter is a critical prerequisite in the design and establishment of manageable procedures to improve plant productivity under different environmental scenarios.

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# 8 Plant Hormone Functions in Abiotic and Biotic Stress Responses

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## 8.1 INTRODUCTION

Plants have evolved complex systems of defense against variable and often potentially damaging environmental conditions. These systems involve mechanisms to sense stresses and to generate and transduce appropriate stress signals, which result in modulation of transcription profiles, leading also to appropriate changes in growth and development. Character of the individual responses depends on the type of stress, its strength and duration, as well as on the physiological state of plant and its strategy to cope with the particular stress.

The growth and development of plants, as well as their responses to changing environmental conditions, are, at least partially, regulated by phytohormones. Two major groups of classical hormones may be recognized: “stress hormones,” including abscisic acid (ABA), ethylene, jasmonic acid (JA) and salicylic acid (SA) and “positive growth regulators,” such as auxins, cytokinins (CK), gibberellins (GA), and brassinosteroids (BR). The former group mediates activation of the defense pathways, whilst the latter ones promote cell division and growth, affecting plant vigor and stimulating plant development. The characteristic feature of “stress hormones” is inhibition of cell division, which is in accordance with the growth suppression occurring frequently under stress conditions, in order to allow the reallocation of limited resources to energy costly defense responses (Lopez et al., 2008). Prolonged stress stimulates change of carbon fluxes from the primary to secondary metabolic pathways, inducing a shift of available resources in favor of a synthesis of secondary products (Iriti and Faoro, 2009).

## 8.2 PLANT HORMONES

### 8.2.1 STRESS HORMONES

#### 8.2.1.1 Absciscic Acid

Absciscic acid (ABA) is the key hormone in the response to abiotic stresses associated with dehydration apart from drought, salinity, and cold stresses. ABA confers resistance to dehydration not only in the stress conditions, but also in specific developmental stages, namely, in seed desiccation.

ABA is a 15-carbon terpenoid, formed by cleavage of 40-carbon precursor, synthesized from isopentenyl diphosphate via plastid terpenoid pathway (Finkelstein, 2006). ABA is synthesized in almost all cells that contain plastids. The key regulatory enzyme is NCED (9-*cis*-epoxycarotenoid dioxygenase). ABA can be deactivated by oxidative degradation or glucoconjugation.

ABA mediates both fast responses (stomata closure) and long-term changes in the expression of many stress-associated genes. As stomata are the place of predominant water loss, precise regulation of their aperture is of high importance at the conditions of water deficit. Stomata closure is caused by loss of turgor of the guard cells by ion efflux.

The first signal sent by the roots exposed to water deficit is alkalization of xylem sap, which allows more ABA molecules to reach the guard cells. At the higher xylem pH, ABA, as a weak acid, occurs preferentially in non-dissociated form, which cannot diffuse across the plasma membranes of mesophyll cells. Thus, much more ABA molecules can reach the stomata cells. Later on, stimulation of ABA biosynthesis, hydrolysis of its glucoconjugates, as well as elevation of ABA transport from the roots take place. ABA signal transduction starts with its binding to PYR/PYL/RCAR receptors proteins, which enables interaction with protein phosphatase ABI1 (PP2C). The formation of this complex results in derepression of kinase SnRK2.6/OST1 and activation of anion channel SLAC1.

After ABA binding to receptor, signal transduction is activated. The network involves production of reactive oxygen species (ROS), NO, elevation of intracellular  $\text{Ca}^{2+}$ , and activation of ion channels (outward transport of  $\text{K}^+$  and  $\text{Cl}^-$ ). The second messengers were identified as sphingosine-1-phosphate, inositol 1,4,5-triphosphate, myo-inositol-hexaphosphate, cyclic ADP-ribose, and phosphatidic acid. Stimulation of ABA signaling results in the expression of transcription factors (MYB, MYC, ABI5), which stimulate expression of the stress-inducible genes. ABA up-regulates expression of LEA proteins, proteases, chaperonins, ion and water channels, antioxidant enzymes, and enzymes involved in metabolism of compatible solutes (Finkelstein, 2006). Apart from the direct effect on gene expression, ABA can affect many posttranscriptional modifications (e.g., formation of cap-binding complex, transfer of mRNAs to nuclear speckles, stimulation of mRNA degradation by microRNAs) (Shinozaki and Yamaguchi-Shinozaki, 2007).

Binding of ABA to receptor FCA (FLOWERING CONTROL LOCUS A, an RNA-binding protein), localized in the nucleus, enables accumulation of mRNA for FLC (repressor of flowering), which prevents transition from vegetative to reproductive phase (Hirayama and Shinozaki, 2007). Inhibition of this transition may positively affect plant tolerances to stresses (e.g., winter wheat cultivars lose their frost tolerance after the initiation generative development, i.e., flower induction).

The other ABA receptor, ABAR, is localized in chloroplasts. ABAR is a H subunit of Mg protoporphyrin IX chelatase, which plays a decisive role in plastid retrograde signaling, i.e., transfer of the signal from chloroplast to the nucleus, in order to coordinate the expression of photosynthetic genes during plant stress responses (Fernandez and Strand, 2008).

The ABA role in biotic stress responses is not straightforward. ABA-induced stomata closure may limit the pathogen entry into the cells. In later stages of infection, high ABA levels were found to have a rather negative effect, especially in the case of biotroph infection (Robert-Seilanianz

et al., 2007). Potential explanation might be antagonistic relationship among individual signaling pathways. It seems that response to abiotic stress has preference in plants, i.e., that strong stimulation of ABA signal transduction pathway (indicating exposure to severe abiotic stress) may be associated with the suppression of the defense pathways toward pathogens.

Increase of the ABA content or over-expression of transcription factor(s) from ABA signaling pathways was repeatedly reported to enhance tolerance to abiotic stresses. Pretreatment of rice with ABA enhanced its tolerance to lead (Zhao et al., 2009). Elevation of ABA content by over-expression of the gene for the key ABA biosynthetic enzyme (*SgNCEDI*) in tobacco enhanced its tolerance to drought and salt stress, which was associated with elevated content of  $H_2O_2$  and NO, as well as of antioxidant enzymes Cu/Zn superoxide dismutase (SOD), catalase, ascorbate peroxidase, and glutathione reductase (Zhang et al., 2009b). Over-expression of *AtMYB96* increased drought tolerance in Arabidopsis, reducing simultaneously lateral root growth (Seo et al., 2009).

### 8.2.1.2 Ethylene

Ethylene is a hormone involved in response to biotic as well as abiotic stresses, playing an important role in stimulation of the expression of defense genes, leaf and fruit abscission, and programmed cell death (PCD). Apart from stress functions, ethylene regulates fruit ripening and determines the onset of natural as well as drought-induced senescence (Young et al., 2004). Ethylene has a negative effect on the photosynthetic performance of leaves.

Ethylene is the only gaseous hormone. It is formed in most organs of higher plants. Its transport through the tissues is very fast and easy. The ethylene precursor is methionine, converted to S-adenosylmethionine. The rate-limiting step is the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. Ethylene biosynthesis is stimulated by stress, certain physiological processes (e.g., ripening of climacteric fruits), or auxin.

Ethylene has a characteristic triple response on etiolated seedlings, i.e., inhibition of hypocotyl growth and its radial swelling, exaggerated curvature of the apical hook, and down-regulation of root growth (Ecker, 1995). Due to this specific response, a number of mutants were isolated, which allowed identifying components of ethylene signaling pathway. Ethylene receptors are associated with the membrane of endoplasmatic reticulum. In the absence of ethylene, they interact with CTR1 kinase, repressor of ethylene signaling (Kieber et al., 1993). Ethylene binding to receptors requires a copper cofactor provided by copper transporter RAN1 (Hirayama et al., 1999). After ethylene binding, CTR1 is released from the complex with receptors, which results in the loss of its activity. Function of at least one ethylene receptor (ETR1) is regulated by another membrane protein RTE1/GR. Ethylene signal is transferred by phosphorelay to transmembrane protein EIN2 (Li et al., 2009) and then to transcription factors EIN3 and EIL1, which results in increase of their stability (Li and Guo, 2007). The strength of the signal is regulated by two F-box proteins EBF1 and EBF2, which control degradation of EIN3 and EIL1 by proteasome (Kendrick and Chang, 2008). As transcription of EBF2 is up-regulated by ethylene, this protein represents a negative feedback loop. EIN3 and EIL1 activate expression of a number of ethylene-induced genes, e.g., transcription factor ERF1, which converges ethylene and JA signaling pathway, or ERF3, involved in signaling pathway of ABA, SA, and JA.

Stimulation of ethylene production was found in drought as well as in flooding, cold, heat stress, salt, ozone, infection (especially with necrotrophs), and mechanical wounding. Stimulation of ACC synthesis and subsequently of ethylene was found in drought, when ethylene accelerated the senescence of the oldest leaves (Apelbaum and Yang, 1981). Increase of transcription of genes involved in ethylene biosynthesis was detected after flooding (Komatsu et al., 2009). It is interesting that ethylene production was stimulated after waterlogging in both susceptible and tolerant *Chrysanthemum* cultivars; however, ethylene peak occurred 2 days earlier and was 3 times higher in tolerant line. Simultaneously, antioxidant enzymes (SOD, ascorbate peroxidase and catalase)

were stimulated (Yin et al., 2009). Ethylene increases the tolerance to salinity stress by down-regulation of the growth via stabilization of DELLA proteins (Li and Guo, 2007).

Ethylene regulates entry into several types of PCD (Young et al., 2004). PCD was observed after ozone application, high light stress or wounding. Ethylene-induced PCD is involved in aerenchyma formation (Trobacher, 2009). PCD stimulated by ethylene is an important component of the hypersensitive response (HR) to pathogen infection, i.e., for the stimulation of the defense not only at the place of infection, but also at the distant tissues. Coordinated effect of ethylene and JA is necessary for initiation of the HR to necrotroph infection. Necrotrophs are pathogens that do not need living plant cells for their growth and multiplication. The other group of pathogens—biotrophs—require and utilize living plant cells for their nutrition.

Ethylene interacts with SA during HR to biotrophic pathogens (Mur et al., 2009). The first transient peak of ethylene was detected 1–4 h after inoculation with HR-eliciting strain. The second, avirulence-dependent rise of ethylene was found after 6 h (only in HR-eliciting strains). Oxidative stress or SA initiated monophasic ethylene generation, while NO was necessary for the biphasic pattern. Also, the response to ozone exhibited biphasic regulation of ethylene formation. Ozone application resulted in rapid (within 1 h) up-regulation of the expression of ACC synthase gene (*ACS6*), ACC oxidase genes (*ACO1*, *ACO3*) as well as of *ETR1* and *ETR2* (receptors) in tomato plants (Moeder et al., 2002). This stimulation was only transient, down-regulated by negative feedback. Later on, the second stimulatory phase occurred. The expression pattern of ethylene co-localized with increase of  $H_2O_2$  after 7 h and necrotic lesions (indication of cell death) after 24 h. In tobacco, ozone application was reported to induce within 90 min peak of NO, which caused rapid  $H_2O_2$  accumulation, followed by an increase in ethylene levels (which might correspond to the second ethylene phase) (Pasqualini et al., 2009). Also Wang et al. (2009) concluded in their study of salt stress response that ethylene was a downstream signal molecule in NO action. Both substances were found to be involved in stimulation of plasma membrane  $H^+$ -ATPase activity involved in control of ion homeostasis (including  $Na^+/K^+$  ratio).

Stimulation of ROS and ethylene signaling resulting in PCD was reported after high light stress (excess excitation energy stress) (Muhlenbock et al., 2008). Dose-dependent elevation of ethylene was also detected at irradiation with UV-B and UV-C (Katerova et al., 2009). Ethylene elevation, stimulation of caspase-like proteases, and subsequent PCD were detected after treatment with cadmium (Iakimova et al., 2009). Similar effect was also exhibited by mercury (Ge et al., 2009). Ethylene plays an important role in leaf and petal abscission, when sensitivity to ethylene is increased only in cells of abscission zone (after down-regulation of auxin transport from the leaf). Ethylene then induces expression of enzymes involved in cell-wall loosening (e.g., cellulase). Leaf abscission is an important mechanism to decrease water loss by diminishing the leaf surface.

Ethylene exhibits an intensive cross-talk with other hormones, predominantly with JA. Antagonistic relationship between ethylene and ABA signaling seems to be the reason for reduced sensitivity of stomata closure to ABA after exposure to ozone, which is associated with ethylene elevation (Wilkinson and Davies, 2009).

Ethylene levels may be increased by incubation in ethylene atmosphere, by application of ethrel, or simply by an incubation with ripe climacteric fruits (e.g., bananas or apples), which emanate ethylene. Over-expression of genes for ethylene biosynthetic enzymes was reported to increase stress tolerance. Also, over-expression of transcription factor *GmERF3* increased tolerance to drought, salt, as well as to diseases (Zhang et al., 2009a).

Down-regulation of ethylene levels may be achieved by inhibition of biosynthetic enzymes (with aminooxyacetic acid or aminoethoxyvinylglycine), ethylene signaling by  $Ag^+$ , which binds to receptors instead of Cu. Antisense expression of genes for ACC synthase and ACC oxidase was found to reduce ethylene evolution after wounding. Down-regulation of ethylene production may improve regeneration of some *in vitro* cultures, preventing accumulation of ethylene to growth inhibitory levels in closed space above the explant. Manipulation of ethylene levels is widely used in controlled fruit ripening and prolongation of fruit shelf life. Sometimes, both stress response and modulation of the developmental process may affect each other. For example, increase of ethylene biosynthesis

in avocado fruits by in-orchard chilling stress was found to speed up fruit ripening, shortening substantially the shelf life of harvested fruits (Hershkovitz et al., 2009).

### 8.2.1.3 Jasmonic Acid

Jasmonic acid (JA) mediates resistance to necrotrophic pathogens and wounding (e.g., by pests). JA is involved in local PCD, which restricts pathogen invasion as well as hypersensitive response. JA was reported to play an important role in the defense to ozone and salinity stresses. JA, as well as ABA, down-regulates cell division, inhibiting cell cycle progression. When applied before G<sub>1</sub>/S transition, JA or ABA prevented DNA replication, keeping the cells in G<sub>1</sub> phase. JA also prevented mitosis, suppressing G<sub>2</sub>/M transition by reduction of histone H1 kinase activity of kinases associated with p13<sup>suc1</sup> protein (Swiatek et al., 2002). JA also exhibits negative effects on photosynthetic performance of the leaves, which allows avoiding potential damage of both photosystems under stress conditions. JA has also important functions in the regulation of plant development, e.g., inhibition of root growth, potato tuberization, fruit ripening or pollen viability, and stimulation of plant senescence (Chico et al., 2008).

JA belongs to a family of oxylipins. Jasmonate biosynthesis starts in plastids, where membrane lipids are cleaved by phospholipases to release  $\alpha$ -linolenic acid. Phospholipase D (PLD) activity during the wounding response was found restricted to the ruptured cells (Bargmann et al., 2009). Linolenic acid is oxidized by lipoxygenase (LOX) to 13(S)-hydroperoxylinolenic acid, which is via 12,13(S)-epoxylinolenic acid further oxidized to 12-oxo-phytodienoic acid (OPDA). After OPDA transport to peroxisomes, JA is produced by repeated  $\beta$ -oxidation.

Synthesis of JA is stimulated by systemin, a proline-rich peptide comprising of 18 amino acid residues. Systemin itself is released after wounding from prosystemin (200 amino acid residues) in parenchyma cells of vascular bundles (Hause et al., 2003). Induced JA stimulates signal transduction in wounded leaves. Simultaneously, JA promotes formation of systemin. This positive feedback loop allows amplification of the signal after wounding or infection.

JA can be metabolized to tuberonic acid (12-hydroxy derivative of epi-JA), which is (as well as its sulfate) a deactivation product. The volatile derivative of JA, its methylester, is also considered as the active form. It may serve as a mobile signal exerting communication among attacked and non-harmed plants. JA forms conjugates with glucose and amino acids, especially with isoleucine (Ile). The key enzyme for this conjugate formation is JAR1. JA-Ile is physiologically active in the signal transduction.

JA-Ile conjugate was found to mediate (like a glue) interaction between JA receptor COI1 (F-box protein of an SCF-type E3-ubiquitin ligase) and JA repressors JAZ (jasmonate ZIM-domain proteins). In *Arabidopsis*, 12 JAZ proteins were found with different tissues and pathway specificity. JAZ binding to COI1 initiates its degradation by proteasome, which releases expression of transcription factors, especially MYC2 (bHLHzip-type), but also ERF1, ERF2, ERF4, MYB21, MYB24, WRKY70, and WRKY18 (Chico et al., 2008). MYC2 positively regulates resistance to insect and herbivory, tolerance to oxidative stress, and flavonoid synthesis. It negatively regulates tryptophan metabolism (Dombrecht et al., 2007). Apart from the stimulation of the defense genes, MYC2 transcription factor also up-regulates expression of JAZ. This negative feedback loop allows precise regulation of the strength of the signal and its effective “switch off” (Chini et al., 2007).

JA-induced genes include proteinase inhibitors, enzymes of flavonoid biosynthesis (chalcon synthase, phenylalanine ammonia lyase, and polyphenol oxidase), sesquiterpenoid biogenesis, and antifungal protein (thionin and osmotin) production (Vasyukova and Ozeretskovskaya, 2009). Expression of genes for proteinase inhibitors was recorded 1–2 h after mechanical wounding or insect attack (Zhao et al., 2003). Proteinase inhibitors inactivate nutrient digestion enzymes in insects. Their knockdown resulted in much faster consumption of leaves by caterpillars. Plants can distinguish between mechanical wounding and insect-induced damage and adjust their defense.

Apart from the huge transcriptomic changes, JA responses involve modulation of ion fluxes across the plasma membrane, generation of reactive oxygen species (ROS) and NO, deposition of callose, activation of calcium-dependent and MAP kinases (Lopez et al., 2008).

Wounding was reported to induce two peaks of JA in wounded leaves and stalks, but only one peak in systemic leaves (Yang et al., 2009). LOX activity (as well as the protein) was induced in the later phase. Wound-induced JA originated in wounded leaves from its biosynthesis and conversion from its conjugates, while in systemic leaves it resulted from transport and biosynthesis via LOX pathway.

Rapid accumulation of JA-Ile (in less than 5 min) was found after wounding in Arabidopsis leaves, distal to the wound site (Koo et al., 2009). Wound-induced systemic production of JA-Ile required JA biosynthetic enzymes (namely, OPDA reductase 3) in undamaged responding leaves. Induced synthesis of JA-Ile correlated with a rapid decline of OPDA, which indicated that transmitted signal triggered systemic synthesis of JA, which upon conversion to JA-Ile by JAR1 activated the expression of early response genes.

#### 8.2.1.4 Salicylic Acid

Salicylic acid (SA) is the key hormone in defense to biotrophic pathogens. Protective properties of SA and of several its derivatives have been recognized for many centuries in human medicine. Hippocrates in the fifth century B.C. recommended bark from *Salix*, natural source of SA, for treatment of cold or fever. Nowadays, a derivative of SA, acetylsalicylic acid, belongs to abundantly used medicines. As a plant hormone, SA governs resistance to biotrophs. SA mediates both the immediate defense against infection and systemic acquired resistance (SAR), which provides a long-lasting protection from further infections by a broad range of biotrophic, hemibiotrophic, and necrotrophic pathogens (Grant and Lamb, 2006). SA suppresses auxin and ABA signaling, which allows diminishing of the availability of nutrients for pathogens as well as relocation of plant resources toward activation of defense compounds (Lopez et al., 2008). SA may enhance tolerance to abiotic stresses by stimulation of antioxidative capacity (Horvath et al., 2007).

SA is an *o*-hydroxyderivative of benzoic acid. It is synthesized in plants from chorismic acid either by isochorismate synthase and isochorismate pyruvate lyase or by phenylalanine ammonia lyase. SA can be reversibly converted into its methylester. Down-regulation of SA levels is achieved by SA decarboxylation.

Crucial positive component of SA signal transduction pathway is NPR1 protein (nonexpressor of pathogenesis-related genes1). Non-induced form of NPR1 is an oligomer. Upon SAR induction, NPR1 is reduced and transported in monomeric form to nucleus, where it activates gene expression (Mou et al., 2003). SA signaling pathway has NPR1-dependent and NPR1-independent branches (stimulation of 193 and 24 genes in Arabidopsis, respectively, Despres et al., 2000). Immediately downstream of NPR1 is activation of the subclass II of TGA transcription factors, TGA2, TGA5, and TGA6 (Zhang et al., 2003). Other primary targets of NPR1 are genes coding ER proteins of secretory machinery and WRKY transcription factors. SA was found to induce activity of phospholipase D (within 45 min); this stimulation is upstream of induction of WRKY38 (Krinke et al., 2009). SA-activated transcription factors include these groups: NAM, ERF/AP2, and MYB. Another early response gene is phosphatidylinositol 4-kinase (Krinke et al., 2007).

Some of the best characterized NPR1-dependent genes are coding pathogenesis-related proteins (PR-proteins), especially PR-1, PR-2, and PR-5 (Srinivasan et al., 2009; Blanco et al., 2009). PR-proteins represent important components of plant defense against pathogens. Recently, 17 families of PR-proteins have been classified. Their expression and accumulation is very specific, precisely associated with the type of pathogen, type of plant cells, and biosynthesis and activation of signaling compounds, i.e., SA, JA, and/or ethylene (Byczkowski et al., 2009). Many PR proteins occur in plants constitutively (although at low concentration). Some of them are specific for certain developmental stages or for specific tissues.

SA-binding protein SABP2 was identified as methyl salicylate esterase. This enzyme is inhibited by its product—SA (Forouhar et al., 2005). SABP2 is required for SAR signal perception in systemic tissue (Park et al., 2007a). The data indicate that MeSA is a long-distance signal in SAR, produced from SA by SA methyltransferase in primary infected leaves and released back to SA in the systemic ones. In *Arabidopsis*, five genes for MeSA-esterases were recently found (Vlot et al., 2008). Three of them, AtMES1, 7, and 9 were found transcriptionally up-regulated during infection with avirulent strain of *Pseudomonas syringae*.

SA plays additional roles in defense and acclimation responses to stresses, such as intracellular stress signaling, improvement of pathogen recognition, and promotion of metabolic changes (Blanco et al., 2009). Upon infection, levels of SA and its glucosylconjugate increase, both in infected and systemic leaves. SA accumulation is induced by  $H_2O_2$ , suggesting a link between the oxidative burst observed during hypersensitive reaction and induction of SA-mediated signal transduction (Summermatter et al., 1995). Positive feedback interaction between SA and ROS seems to be associated with PCD (Overmyer et al., 2003). SA is also involved in the control of cellular redox balance at the onset of SAR.

SA role in various abiotic stress responses seems to be based on its ability to stimulate the activity antioxidant system, which improves integrity of cellular membranes and facilitates sustainment of photosynthesis and of general metabolism (Farooq et al., 2009). SA activates genes coding for detoxifying or antioxidant enzymes. The first characterized SA-binding protein was identified as catalase. Another SA-binding protein, SABP3, was found in the chloroplast stroma (Slaymaker et al., 2002). It was identified as carbonic anhydrase with antioxidant activity. SA pretreatment was reported to increase the tolerance to cold stress, as well as to heavy metals by stimulation of the antioxidant enzymes ascorbate peroxidase and Cu/Zn superoxide dismutase (Horvath et al., 2002; Krantev et al., 2008). SA was also reported to enhance activity of glutathion S-transferases and glucosyltransferases (Blanco et al., 2009).

SA was reported to be active also in the defense against rice blast (Bailey et al., 2009), in spite of the fact that JA pathway, together with ethylene, mediates rice defense gene expression. It was found that SA acts here as a constitutive antioxidant, protecting rice from the pathogen-induced oxidative damage.

Plants and pathogens have continuously confronted each other during evolution in a battle for growth and survival (Lopez et al., 2008). The antagonistic relationship between SA and JA signaling pathway has been utilized by pathogen *Pseudomonas syringae*, which produces JA-Ile analogue, phytotoxin coronatine. This phytotoxin is able to stimulate JA signaling pathway, which results in the suppression of the SA pathway (Brooks et al., 2005). It is interesting that coronatine also facilitates bacterial invasion by repression of ABA-mediated stomata closure (Melotto et al., 2006).

Activation of plant defense signaling pathways depends on the pathogen lifestyle and its mode of infection. Some pathogenic fungi secrete mannitol into the apoplast to suppress ROS-mediated host defense. Part of SA-induced defense is secretion of symplastic plant enzyme mannitol dehydrogenase into the apoplast, where it converts mannitol to mannose (Cheng et al., 2009).

Exposure to stress (nonlethal) usually increases tolerance to subsequent stresses, mainly due to the maintenance of the concentrations of certain components of defense pathways up-regulated above their basal levels (for certain period), which makes the response to another stress (even of different kind) faster and more efficient. On the contrary, simultaneous exposure to different stresses has usually a negative impact, as different signaling pathways affect each other in an antagonistic way (as mentioned above in case of ABA and JA or SA signaling). Thus, exogenous SA at low concentration (less than 0.25 mM) enhanced drought tolerance of barley leaves, elevating the expression of dehydrin gene, as well as the level of the corresponding protein, decreasing the electron leakage and malondialdehyde and hydrogen peroxide content (Sun et al., 2009). High SA concentration (above 0.5 mM) had negative effects, suppressing the accumulation of dehydrin proteins and aggravating the oxidative damage.

Modulation of SA levels was used for the study of the mode of action of this hormone. Down-regulation of SA levels was achieved by over-expression of the gene for SA decarboxylase.



Stimulation of SA signaling by over-expression of specific signaling components was reported to enhance tolerance to different stresses. For example, over-expression of *NPR-1* had positive effect on SA-mediated response to fungal and bacterial pathogens, but negative effect on the tolerance to dehydration and salt stresses as well as viral infection (Quilis et al., 2008).

## 8.2.2 POSITIVE GROWTH REGULATORS

### 8.2.2.1 Auxin

Auxin is a crucial hormone for regulation of plant growth, orchestrating many developmental processes, especially embryogenesis, differentiation of vascular tissues, and lateral root initiation (Woodward and Bartel, 2005). Auxin is indispensable for cell division and elongation. It exhibits its multiple physiological effects, including apical dominance, stimulation of root growth, tropic responses, and prevention of abscission.

The most extensively studied endogenous auxin is indole-3-acetic acid (IAA). IAA is synthesized by several tryptophan-dependent and independent pathways. IAA is formed primarily in shoot and root apices, but also in leaves (especially, the young ones), cotyledons, and roots. There are two main pools of auxin in the cells: the cytosol and chloroplasts. IAA can be reversibly converted to indole-3-butyric acid (IBA). The levels of free IAA are regulated by conjugation with sugars, amino acids, and peptides. Levels of IAA are also down-regulated by its degradation, compartmentation, and polar auxin transport. Auxin polar transport represents an important mechanism in its mode of action. It is regulated predominantly by asymmetrical localization of auxin efflux proteins, PINs, in the cells (Zazimalova et al., 2007).

Auxin signaling pathway involves formation of a complex between receptors TIR and repressors of Aux/IAA family. Auxin mediates (like a glue) their binding, which stimulates degradation of the repressor molecules by proteasome. After repressor removal, positive ARF proteins can stimulate expression of auxin-responsive genes (Tiwarei et al., 2004). The early auxin-responsive genes include transcription factors, genes involved in stress adaptation, and intercellular signaling. Auxin rapidly and transiently induces accumulation of at least three families of transcripts: SMALL AUXIN-UP RNAs (SAURs), GH3-related transcripts (coding enzymes involved in IAA-amino acid conjugation), and those coding repressor AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins (Woodward and Bartel, 2005). Stimulation of the expression of Aux/IAA genes by auxin (resulting in accumulation of new repressors) ensures transient character of auxin signal.

As mentioned above, IAA is a strong positive regulator of cell division and growth. Its high content in shoot apex and young leaves coincides with relatively high growth rate. Many abiotic stresses are associated with the suppression of the shoot growth, which is in good correlation with the decrease in free IAA content in apex and upper leaves observed, e.g., in drought-stressed tobacco (Havlova et al., 2008). Modulation of free IAA content involves stimulation of *GH3* genes for auxin-conjugating enzymes. Park et al. (2007b) suggested that GH3-mediated down-regulation of IAA levels, resulting in growth suppression, directs reallocation of metabolic resources to defense pathways. Also cold stress, both sudden and with previous acclimation, have been associated with down-regulation of auxin levels by strong increase of GH3-like proteins (Meng et al., 2008). Auxin signaling was found down-regulated, e.g., by cold-inducible microRNAs (Zhou et al., 2008).

Very prominent feature of abiotic stress responses is stimulation of IAA accumulation in roots. Several reports (Ribaut and Pilet, 1994; Xin et al., 1997; Havlova et al., 2008) described significant increase of IAA in roots of drought-stressed plants. Auxin accumulation in roots might allow maintenance of the root growth resulting in the elevation of the root/shoot biomass ratio. Simultaneous elevation of ABA (Seo et al., 2009) and cytokinins (Havlova et al., 2008) seemed to prevent lateral root formation. Initiation of lateral roots requires local auxin maxima. Drought-enhanced ABA stimulated expression of MYB96 transcription factor, which down-regulated auxin levels via induction of *GH3* genes at the sites of potential lateral roots initiation (Seo et al., 2009). The resulting effect is stimulation of the primary root growth, which allows the plant to

reach deeper soil levels obtaining access to underground water. Relatively high CK levels in stressed roots might also contribute to this phenomenon.

Similar changes as drought, i.e., strong down-regulation of IAA in the leaves and accumulation in roots, were imposed by salinity (100 mM NaCl, 3 weeks) (Albacete et al., 2008).

Apart from its morphological effects, accumulation of auxin in stressed roots may have protective functions. Auxin application was found to induce Cu/Zn SOD and catalase transcripts in the distal root zone and peroxidases in the whole root. Simultaneously, levels of H<sub>2</sub>O<sub>2</sub> were diminished (Tyburski et al., 2009). The positive effects of auxin on antioxidant system may be involved also in the response to heavy metal stresses. Exposure to arsenic induced in the stress-tolerant variety of *Brassica juncea* L. fast (within 6 h) transcriptome changes, which included up-regulation of auxin and JA biosynthesis and down-regulation of ethylene biosynthesis and CK-responsive genes. This coordinated hormonal response was not found in the sensitive variety (Srivastava et al., 2009). Stimulation of the expression of auxin-responsive protein (SdARP) upon mercury stress was observed in the heavy metal hyperaccumulator *Sesbania drummondii* (Venkatachalam et al., 2009). Also, the response to aluminum stress involved up-regulation of auxin-responsive genes, together with genes for peroxidases, chitinases, and GSTs (Goodwin and Sutter, 2009). Genes involved in maintenance of chloroplast structure and photosynthesis were down-regulated. Toxic levels of aluminum were found to suppress root growth, mainly by blocking auxin transport via inhibition of the transport of PIN2 containing vesicles from plasma membrane to endosomes (Hong et al., 2009).

IAA plays an important role in delay of abscission. Auxin flow from the (stress-induced) senescing leaves is necessary to diminish the sensitivity of cells from abscission zone to ethylene. After auxin flow cessation, the ethylene sensitivity increases and abscission starts. Delay in abscission might contribute to relocation of nutrients from senescing leaves before their final death. Increase in free auxin levels in senescing leaves by nitrilase 2, an enzyme mediating the synthesis of IAA from indole-3-acetonitrile, was described by Quirino et al. (1999).

Function of auxin in biotic stress response depends on the type of invading pathogen. Some biotrophs enhance nutrient and water supply from the host by modulation of plant-hormone production and/or signaling. They can produce hormones themselves, as a component of their invading strategy (Lopez et al., 2008). For example, *Moniliophthora perniciosa*, which colonizes apoplast as biotroph pathogen causing “Witch’s broom” disease, is able to produce both auxin and gibberellins (Mondego et al., 2008). Plants defend to biotrophs by down-regulation of auxin signaling. Elevation of the content of miR393, involved in repression of auxin signaling, contributed to resistance against *Agrobacterium tumefaciens* (Pruss et al., 2008).

Some bacteria, which produce auxin, can be, however, beneficial, for plants. IAA-overproducing strain of *Sinorhizobium meliloti* improved the growth of *Medicago truncatula* by elevation of the IAA content in roots and nodules (Bianco and Defez, 2009). Nodulated plants had enhanced salt tolerance, which coincided with stimulation of the activity of antioxidant enzymes (SOD, peroxidases, glutathion reductase and ascorbate peroxidase).

### 8.2.2.2 Gibberellins

Gibberellins (GAs) were first isolated from a filtrate of pathogenic fungus *Gibberella fujikuroi*, which caused tall growth of rice plants, reducing their seed production. Later on, they were identified also as plant hormones. GAs regulate numerous developmental processes, especially seed germination, stem elongation (of rosette plants), leaf expansion, and stimulation of flowering. They inhibit nodulation and tuberization. Until now, more than 136 GAs have been found, but only few are physiologically active (GA<sub>4</sub> and GA<sub>1</sub>).

GAs are diterpenes synthesized from geranylgeranyl diphosphate. The key biosynthetic enzymes are GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). GA inactivation is achieved either by their 2-hydroxylation by GA 2-oxidase or by GA glucosylation. Recently, other GA deactivation enzymes were reported: cytochrome P450 monooxygenase and GA methyltransferase (Zhu et al., 2006; Varbanova et al., 2007).

GA signaling pathway is strongly down-regulated by repressors DELLA proteins (Zentella et al., 2007), which require for their full activation function of O-linked *N*-acetylglucosamine transferase SPY (SPINDLY, Maymon et al., 2009). Binding of GA to soluble receptor GID1 enables its interaction with DELLA proteins and targeting repressors to 26S proteasome degradation. Removal of repressors allows stimulation of the expression of MYB transcription factors and subsequent GA responses, e.g., up-regulation of  $\alpha$ -amylase genes.

It seems that tolerance to abiotic stresses coincides with down-regulation of active GA levels or suppression of sensitivity to GAs (e.g., at heat stress, Sarkar et al., 2004). Repression of *GA3ox1* gene was found under strong salinity stress (Kim et al., 2008). UV-B exposure was found to decrease endogenous GA levels via induction of genes involved in GA deactivation: *GA2ox2* and *GA2ox8* (Ulm et al., 2004). Salinity was reported to result in stimulation of the expression of 6 genes for GA 2-oxidases (Magome et al., 2008). Expression of *GA2ox7* was in Arabidopsis up-regulated by DDF1 (dwarf ABD delayed flowering 1), an AP2 transcription factor of the DREB1/CBF subfamily. Under high salinity, DDF1 not only affected GA synthesis, but also GA signaling by up-regulation of DELLA protein RGL3 (Kim et al., 2008). Stabilization of DELLA proteins coincides with GA down-regulation and results in stimulation of the expression of genes for ROS-detoxifying enzymes (Achard et al., 2008a). DELLA proteins contribute significantly to cold acclimation and freezing tolerance (Achard et al., 2008b). They restrain cell production by enhancing levels of the cell cycle inhibitors Kip-related protein 2 (KRP2) and SIAMESE (SIM) (Achard et al., 2009).

GAs also negatively regulate accumulation of transcription factor GhDREB1, which is induced by low temperature and salt stress. Tobacco plants over-expressing *GhDREB1* displayed stronger chilling tolerance, maintaining higher net photosynthesis rate and proline content than WT (Shan et al., 2007). Under optimal conditions, they, however, display retarded growth and delayed flowering.

When the effect of osmotic stress (achieved by 12% PEG application) on hormone pools was followed, down-regulation of GAs was accompanied by decrease of CKs, while transient stimulation was observed in case of IAA and high up-regulation in case of ABA (Wang et al., 2008). It is interesting that growth of roots was much less retarded than that of shoots.

Negative effect of heavy metals (Hg, Pb) on flowering time could be partially overcome by exogenous GA<sub>3</sub> application (Chaudhry and Khan, 2006).

GAs have strong stimulatory effect on seed germination. Their exogenous application was repeatedly found to promote seed germination even under unfavorable stress conditions (Alonso-Ramirez et al., 2009). Exogenous GA in combination with CKs enhanced longevity of flowers and leaves of pot tulips (Kim and Miller, 2009).

### 8.2.2.3 Brassinosteroids

Brassinosteroids (BR) are polyhydroxylated steroids, which stimulate growth of both shoots and roots, positively affect cell elongation (in synergism with auxins), cell division (stimulating cyclin D3, similarly to CKs), lateral root growth, promote xylem differentiation, seed germination, pollen tube growth, photomorphogenesis and, especially, stress tolerance (Chory, 2006). BR strongly enhance tolerance to various stresses: cold, heat, salt, drought, high light, heavy metals and viral, bacterial, and fungal infections (Nakashita et al., 2003).

BRs are synthesized via terpenoid pathway from the same precursor as ABA, CKs, and GAs (isopentenyl diphosphate). Two farnesyl diphosphate molecules form squalene, which may cyclise to cycloartenol. Important intermediate of BR synthesis is campesterol, metabolized in several steps to bioactive castasterone and the most active BR brassinolide. BR are deactivated by 26-hydroxylation. The highest level of BRs is in the apical shoot.

BR signaling pathway involves binding of BR to plasma membrane receptor BRI1, which subsequently interacts with coreceptor BAK1 (BRI1-associated receptor kinase 1) (Li and Nam, 2002). BR signal transduction results in deactivation of the repressor kinase BIN2. In the absence of BR, BIN2 phosphorylates transcription factors BES1 (bri1-EMS-suppressor1) and BZR1 (brassinazole-resistant 1), which targets them to proteasome degradation (Belkhadir and Chory, 2006). In the

presence of BR, BES1 and BZR1 are dephosphorylated with Ser/Thre phosphatase BSU1, which enhances their activity. The active BES1 is associated with another transcription factor BIM1 (BES1-interacting Myc-like 1) and binds to the BR-response element in promoters of BR-inducible genes, stimulating their expression. BZR1 binds to promoters of BR biosynthetic genes and represses them. This gene suppression represents negative feedback loop. BRs were reported to induce the expression of heat shock proteins (HSP83, HSP70), heat shock factors (HSF3), and oxidative stress-related genes—GST, ATPA2 and ATPA24a (Goda et al., 2002; Mussig et al., 2002). BR-induced transcription factors are, e.g., WRKY6, CBF/DREB, MYB (e.g., AtMYB30), and MYC. Further BR-induced genes include those coding MAPK1 and MAPK3, phenylalanine ammonia lyase, catalase, ascorbate peroxidase, SOD and glutathione reductase, as well as PR-1 (Xia et al., 2009).

Model for BR induction of stress tolerance was recently presented by Xia et al. (2009). Perception of BR by receptors results in the activation of plasma membrane-bound NADPH oxidase, which may release  $H_2O_2$  into apoplast. Hydrogen peroxide then functions as a signal molecule activating stress response pathways.  $H_2O_2$  levels were found elevated 3 h after BR application and returned to basal level after 3 days.

The activated pathways involve MAPK and stimulation of antioxidant system. BR action was found unrelated to SA signal transduction pathway or to SAR (Robert-Seilanianantz et al., 2007).

Exogenous application of BRs was found to diminish significantly adverse effects of various stresses. Foliar spray of BR was reported to increase drought tolerance in rice, improving net  $CO_2$  assimilation, water use efficiency, leaf water status, membrane properties, free proline content, anthocyanins, and soluble phenolics (Farooq et al., 2009). Uncontrolled increase of  $H_2O_2$  and membrane lipid peroxidation was diminished. Pretreatment with 24-epibrassinolide significantly reduced severity of *Fusarium wilt* infection, improving plant growth (Ding et al., 2009). BRs were reported to increase the yield of bean seeds or lettuce biomass. Their positive effects may be recognized mainly under stress; under optimal conditions, they show only little effect (Ikekawa and Zhao, 1991).

#### 8.2.2.4 Cytokinins

Cytokinins (CKs) were defined as substances stimulating (in the presence of auxin) cell division (cytokinesis) in tissue cultures (Miller et al., 1955). They are indispensable for cell cycle  $G_2/M$  transition (Laureys et al., 1998; Dobrev et al., 2002). They also positively affect  $G_1/S$  transition, stimulating the level of CycD3, which can be, however, increased also by other substances, e.g., sucrose (Riou-Khamlichi et al., 1999). CKs exhibit multiple physiological functions: stimulate differentiation of chloroplasts and positively affect photosynthesis by stabilization of the structure and function of the photosynthetic machinery (Chernyadev, 2009), delay senescence, open stomata, enhance the sink strength (Skoog et al., 1965), and stimulate branching. CKs play a critical role in balancing acquisition and distribution of macronutrients (Hirose et al., 2008). This effect might be associated with CK stimulation of invertase activity (Roitsch and Ehness, 2000).

Natural CKs are adenine derivatives with either isoprenoid or aromatic side chain. The key enzymes of isoprenoid CK biosynthesis are isopentenyltransferases (IPT), which catalyze reaction of isopentenyl diphosphate with ATP (or ADP) (Takei et al., 2001; Kakimoto, 2001; Kamada-Nobusada and Sakakibara, 2009). Down-regulation of CK levels is governed by cytokinin oxidases/dehydrogenases, which cleave the side chain. The other important mechanisms of suppression of CK activity are their glycosylation, irreversible (at the adenine ring in position N7- or N9-) or reversible (O-glucosylation of the side chain or, quite rare, N3-glucosylation, Mok and Mok, 2001). The highest CK content is in root apex. High CK concentrations can be detected at lateral root tips and in shoot apex. CKs are also present in the stem and in leaves (in inverse correlation with the leaf age), especially along the vasculature.

Three CK receptors (AHK2, 3, and 4) differ in their specificity as well as affinity to individual CK bases or, to a lower extent, to ribosides (Spichal et al., 2004; Mok et al., 2005). Structural variation of

the side chain mediates different biological messages, *trans*-zeatin being predominant in regulation of cell division (Hirose et al., 2008). CK signal is transferred by phosphorelay via AHP proteins to type-B response regulators, and by transcription factors which stimulate CK early response genes or negative type-A response regulators, important for “switching off” of the signal. The signal is modulated also by transcription factors, CRFs (Rashotte et al., 2006; Argueso et al., 2009).

Endogenous CK levels are strictly regulated during the abiotic stress responses, both in time- and tissue-specific manner. CKs have an important role in drought response, ensuring preferential protection of shoot apex by elevation of the sink-source polarization of plants in favor of younger leaves (Cowan et al., 2005). The establishment of gradient of active CKs in favor of upper tobacco leaves was found already in mild stress, when it was promoted by elevation of active CKs in upper leaves (Havlova et al., 2008). During drought stress progression, when the levels of active CKs in shoots substantially decreased, their gradient was maintained by stimulation of the activity of cytokinin oxidases/dehydrogenase in lower leaves.

CK levels were much less affected in drought-stressed roots, which might contribute to the maintenance of the root growth, resulting in the increase of the root/shoot ratio. Relatively high CK levels might be involved, together with ABA, in the suppression of lateral root formation.

CKs seem to have an important function at the initial phase of heat stress, when enhanced evapotranspiration was reported to be necessary for cooling of leaf surface (Zhang et al., 2008). CK transient increase, positively affecting stomata aperture, might contribute to transient stomata opening (Vankova, unpublished results).

Plants usually respond to mild and/or short stresses by enhanced metabolic activity, which seems to be associated with increased CK levels. Tran et al. (2007) demonstrated up-regulation of the expression of genes for all CK receptors within 10 min of dehydration. Expression of *AHK2* was also stimulated by NaCl and ABA, *AHK3*, and also by high salinity and cold stress. We found stimulation of genes for all CK receptors within 15 min of heat stress (Vankova, unpublished results). These data are in accordance with reports indicating that exogenous CKs increase tolerance to mild stress and speed up recovery (Itai et al., 1978), as well as positively affect recovery of stomatal conductance and net photosynthesis after rehydration (Rulcova and Pospisilova, 2001). Moreover, transcription of many stress-induced genes could be stimulated by CKs (Hare and Van Staden, 1997). Within 2 h, exogenous CKs were found to up-regulate expression of transcription factors, signaling proteins, developmental and hormonal regulators modulate primary and secondary metabolism; and stimulate energy generation (Brenner et al., 2005).

Prolonged and more severe stress usually coincides with decreased CK levels and growth suppression. While low levels of salt were reported to have positive effect on growth, high salt stress (100 mM NaCl, 3 weeks) led to growth suppression and progressive decrease of active CKs (Ghanem et al., 2008). Photosystem II efficiency started decreasing at the second week of salinization in the middle leaf, and at the end of the third week in the younger one (which indicated protection of younger leaves) (Ghanem et al., 2008).

When ozone-sensitive and ozone-tolerant accessions of *Medicago truncatula* were compared (Puckette et al., 2009), tolerant line up-regulated upon ozone exposure ABA- and auxin-responsive genes, as well as those associated with adaptive responses to stress. Down-regulation was observed in the case of photosynthesis-related genes. Sensitive cultivar up-regulated genes associated with oxidative stress, cell growth, translation, as well as CK-response genes. Most genes in sensitive accession were induced at later time periods. The authors concluded that inability to perform an early transcriptional reprogramming might be the cause for inefficient defense response, which may lead to severe oxidative stress. Thus, up-regulation of CK signaling did not appear as an effective strategy in case of severe stress.

As already mentioned in the case of auxins, regulation of endogenous CK levels in the defense to pathogens strongly depends on the type of infection. Part of the invading strategy of biotroph pathogens is biosynthesis of CKs (Lopez et al., 2008) or modulation of CK metabolism and/or signaling in plants. Down-regulation of the cytokinin oxidase/dehydrogenase activity of the host as well as

stimulation of the expression of CK receptors were reported in *Plasmodiophora brassicae* (Siemens et al., 2006). Elevated CK levels allow the pathogens to attract nutrients into the site of infection as well as delay tissue senescence (i.e., keep it metabolically active). Thus, stimulation of CK deactivation and/or down-regulation of CK signaling seem to be an integral part of plant defense.

There are also antagonists of pathogenic fungi, bacteria-producing CKs, which have positive effect on plant growth. For example, inoculation of lettuce plants with *Bacillus subtilis* resulted in elevation of endogenous CKs in plants, which coincided with increase plant growth (Arkhipova et al., 2005).

No necrotroph was found to produce CKs (Robert-Seilanianitz et al., 2007); therefore, in case of necrotrophs, plant response may involve enhanced CK production. One of the reasons might be stimulation of photosynthesis and enhanced energy formation. Elevation of physiologically active CK (isopentenyladenosine) was found upon infection of grapevine with Grapevine virus A and B (Vankova, unpublished data). Constitutive CK overproduction was found to delay/diminish progression of virus PVY<sup>NTN</sup> in tobacco (Muller, personal communication).

In order to keep equilibrium between energy formation and consumption, suppression of growth proved to be an effective strategy. Tolerance to severe drought and salt stresses was enhanced in single *ahk2* and *ahk3* mutants, and especially in the double mutant (Tran et al., 2007). However, positive effects of exogenous CKs on amelioration of the stress impact indicate that maintenance (at least partial) of photosynthetic activity may be beneficial. When the effect of CKs constitutively elevated before the stress initiation was followed, delay in the stimulation of defense mechanisms, e.g., increase in ABA and xanthophyll cycle pigments was observed (Havlova et al., 2008; Haisel et al., 2008). Strong overproduction of CKs by over-expression of *pssu:ipt* even enhanced plant sensitivity to drought stress (Synkova et al., 1999). This phenomenon, however, might have been caused also by the impaired root system (CK overproduction results in inhibition of formation of roots, the primary site of their biosynthesis).

Unlike constitutive CK overproduction, targeted elevation of CKs was reported to have positive effect on abiotic stress tolerance. Several authors used *SAG12:ipt* construct. *SAG12* promoter is activated at the onset of senescence in the senescing tissues. *SAG12*-driven increase of CK content then suppresses further senescence. Elevation of CKs down-regulates promoter activity, which prevents CKs from reaching superoptimal levels (high CK levels were reported to stimulate cell death, Mlejnek and Prochazka, 2002).

*SAG:ipt* transformed grass *Festuca arundinacea* Schreb exhibited improved cold tolerance (Hu et al., 2005). *SAG:ipt* Arabidopsis plants showed enhanced survival after flooding (Huynh et al., 2005). Creeping bentgrass (*Agrotis stolonifera* L.) over-expressing *SAG:ipt* showed enhanced tolerance to heat stress, exhibiting increase in number of tillers, chlorophyll retention, and root elongation. Recently, significant increase in drought tolerance was reported in tobacco over-expressing *ipt* under *SARK* (senescence-associated receptor protein kinase) promoter (Rivero et al., 2007). This promoter is activated at the very onset of senescence, prior to any visible symptoms. *SARK:ipt* plants exhibited higher WUE and better recovery after drought. They preserved the structure of chloroplasts better than WT and the rate of their photosynthesis was, under drought stress, less inhibited than in WT (Rivero et al., 2009).

Different strategies thus can be applied to diminish the negative impact of stresses: either suppression of growth and stimulation of the synthesis of protective substances or (relative) increase in photosynthesis rate to ensure supply of energy, even under unfavorable conditions. The latter strategy seems to be more promising where the crop yield is concerned. However, it should be kept in mind that in case of very strong and prolonged stresses, it might be detrimental.

Expression of *SAG:ipt* may exhibit a high potential practical impact, e.g., in delay of the postharvest senescence of cassava tuberous roots, prolonging substantially their shelf life (Kaminek, personal communication). It is necessary, however, to keep in mind the physiological consequences of delayed senescence achieved by targeted CK overproduction. Postponed senescence, found highly desirable in case of turfgrass, need not be so positive in case of wheat. Delay of leaf senescence,

achieved by *SAG:ipt* over-expression, substantially prolonged the vegetative period, increasing the sink strength of leaves, which interfered with grain filling (Sykorova et al., 2008).

### 8.3 CONCLUSIONS

Stress physiology represents a dynamically developing area with a wide potential for practical applications. The climate change, coinciding with both increase of the mean temperatures and more frequent extreme events (e.g., storms or floods), as well as the demand to feed increasing world population, indicates the necessity to grow plants in less favorable conditions, enhance stress tolerance of crops, reducing yield losses caused by different stresses and their combinations.

Until now, the most frequent strategy to improve the stress tolerance has been over-expression of transcription factors involved in signaling pathways of stress hormones. Due to the considerable cross talk among the pathways, this strategy may result in enhanced tolerance toward more stresses. However, the consequence of the constitutive activation defense metabolism is usually reduced growth under non-stress conditions. Nevertheless, as the occurrence of stresses is rather frequent and their negative impact on yield rather high, this strategy proved to be useful.

Increasing amount of evidence indicates that ROS may function as a common signal in defense pathways (Apel and Hirt, 2004; Torres and Dangl, 2005). Transient, tightly regulated increase in ROS seems to induce signaling pathways, which involve mitogen-activated kinases, transcription factors, antioxidant enzymes, dehydrins, low-temperature-induced heat shock proteins, and pathogenesis-related proteins (Gechev et al., 2006). These common signals allow increasing tolerance to different stresses by stimulation of the basal level of individual components of the stress-response pathway. Improvement of antioxidant protection of plants, e.g., by enhanced level of BRs, may thus represent a promising strategy for stress-tolerance improvement.

An alternative strategy may be the protection of photosynthesis by elevation of CKs, associated with the suppression of the (stress-induced) senescing process. Thus, targeted elevation of CKs might be useful in elevation of the tolerance to different abiotic and biotic stresses.

The achieved results indicate that modulation of hormone metabolism and/or signaling may represent a promising strategy, especially in combination with suitable tissue- and time-specific promoters.

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# 9 Role of Proline in Plant Response to Drought and Salinity

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## 9.1 INTRODUCTION

Plants face frequent periods of environmental stress that impairs their growth and reproductive capacity. Restriction of plant growth cannot be attributed to one single process, because plant growth is the result of many integrated and regulated physiological and biochemical processes. To survive and maintain minimal growth potential, plants must conform to extreme environmental conditions entailing adaptive changes in metabolism and cell composition. Of the various mechanisms enabling plants to cope with water stress, the most common is the accumulation of intracellular solutes, such as sugars and free amino acids. The most frequent nitrogen-containing compounds that accumulate in plants subjected to drought and salinity are amides, amino acids, and polyamines. The accumulation of proline on dehydration due to water deficit or increasing osmotic pressure is the most recent information concerning the osmoregulatory role of proline in environmentally stressed plants.

Salinity is a permanent threat to crops, especially in countries where irrigation is an essential aid to agriculture. It is estimated that about 20% of cultivated lands and 33% of irrigated agricultural lands worldwide are afflicted by high salinity. Moreover, the salinized areas are increasing at a rate of 10% annually. Droughts are an inevitable and recurring feature of world agriculture, and, despite our improved ability to predict their onset and modify their impact, they remain the single most important factor affecting world food security and the conditions and stability of the land resource from which that food is derived. Drought involves osmotic stress, while salt stress involves both, osmotic stress, by limiting absorption of water from soil, and ionic stress, resulting from high concentrations of potentially toxic salt ions within plant cells. In glycophytes, high salinity reduces growth by inhibiting important physiological processes, such as photosynthesis and nutrient supply to the growing tissues that are directly affected by the reduction of leaf water potential. Nutrient disturbances under both drought and salinity reduce plant growth by affecting the availability, transport, and partitioning of nutrients. Drought reduces both nutrient uptake by the roots and transport from the roots to the shoots, because of restricted transpiration rates and impaired active transport and membrane permeability (Alam, 1999). Under salinity, reduced plant growth is related to specific ion toxicities such as  $\text{Na}^+$  and  $\text{Cl}^-$  and ionic imbalances acting on biophysical and/or metabolic components of plant growth (Grattan and Grieve, 1999). Although increasing the supply of nutrients to the growth medium under drought or saline conditions can alleviate the adverse effects of either stress on plant growth, it is generally accepted that such increases will not improve plant growth when the nutrient is already present in the soil in sufficient amounts and the drought or salt stress is severe. A variety of protective mechanisms have evolved in plants to allow them to acclimatize to these unfavorable environmental conditions for survival and growth. The synthesis and accumulation of low molecular weight metabolites, known as compatible solutes, is a ubiquitous mechanism for osmotic adjustment in plants. Their main role is to increase the ability of cells to retain water without affecting the normal metabolism. Amino acids, sugars, polyols, and various quaternary ammonium compounds are reported to accumulate as compatible solutes in many plant species. Betaines, quaternary ammonium compounds occurring naturally in a variety of plants, animals, and microorganisms and proline are among the most common nitrogen containing compatible compounds. Both have been reported to be involved in the stabilization of proteins and cell structures and/or in scavenging of free radicals, and might serve to store nitrogen that can be used by the plant when the stress is relieved.

## 9.2 OSMOREGULATION OR OSMOTIC ADJUSTMENT

The osmotic potential of cytoplasm is a dynamic cell property that can change when plants are subjected to saline conditions or dehydration, as reported in an early work by Eaton (1927) and subsequently by many other investigators (Morgan, 1984). Salinized plants take up salt from the soil in quantities that decrease the osmotic potential of the cells. Bernstein (1961) termed this change “osmotic adjustment” because the cell osmotic potential paralleled the lowered water potential of the salt-affected soil, thus maintaining a favorable osmotic force for water uptake and a high turgor pressure in the cells. A similar adjustment was later discovered when plants underwent dehydration (Meyer and Boyer, 1972). The term osmotic adjustment is now applied both to dehydrated and salt-affected plants, and is defined as an accumulation or *de novo* synthesis of solute per cell that causes a change in the cell osmotic potential. It excludes changes in cell water content that also can alter cell osmotic potential. Water deficits have been shown to induce a lowering of the osmotic potential in crops as a means of maintaining their turgor (Boggess et al., 1976). This decline in the osmotic potential as a response to water deficit can be achieved by solute accumulation within the plant cell or by a decreased cell volume leading to an increased concentration of osmotic solutes as water leaves from the vacuole. These phenomena are described as osmoregulation and osmotic adjustment. *Osmoregulation* has been defined as “the regulation of osmotic potential within a cell by the addition or removal of solutes from solution until the intracellular osmotic potential is



approximately equal to the potential of the medium surrounding the cell” (Turner and Jones, 1980). *Osmotic adjustment* refers to the lowering of the osmotic potential due to the net accumulation of solutes in response to water deficits or salinity (Turner and Jones, 1980). Osmotic adjustment is an important mechanism in drought tolerance, because it enables (1) a continuation of cell expansion (Hsiao et al., 1976; Wyn Jones and Gorham, 1983), (2) stomatal and photosynthetic adjustments (Ludlow, 1980), (3) better plant growth, and (4) yield production (Morgan, 1983). The compounds involved in osmotic adjustment are mainly soluble sugars, potassium, organic acids, chloride, and free amino acids (Cutler and Rains, 1978; Jones et al., 1980). The degree of the osmoregulatory processes is affected by the rate of stress, stress preconditioning, the organ type, and the genetic variation between and within species (Morgan, 1984). It is accepted that the nontoxic compatible organic solutes accumulate in the cytoplasmic compartment of cells and inorganic ions toxic to metabolic processes are restricted to the vacuolar compartment. Considerable research has been conducted to characterize the accumulation of proline, a compound known to contribute to the osmotic adjustment and tolerance of plants exposed to unfavorable environmental conditions. How much of a role it plays is still controversial and is discussed in detail in the following sections.

### 9.2.1 PLANT RESPONSE TO DROUGHT AND SALINITY

Abiotic stress leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al., 2001). Drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano et al., 1999; Zhu, 2001). Plants respond to osmotic stress caused by drought or salinity at the morphological, anatomical, cellular, and molecular levels (Greenway and Munns, 1980; Bohnert et al., 1995; Yeo, 1998; Hasegawa et al., 2000). These include, for example, morphological and developmental changes such as life cycle, inhibition of shoot growth and enhancement of root growth, adjustment in ion transport through uptake, extrusion and sequestration of ions, and metabolic changes such as carbon metabolism, the synthesis of compatible solutes. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutritional imbalance, specific ion effect, or a combination of these factors (Ashraf, 1994). Some of these responses are triggered by the primary osmotic stress signals, whereas others may result from secondary stresses/signals caused by the primary signals. These secondary signals can be phytohormones, mainly abscisic acid (ABA), reactive oxygen species (ROS), and intracellular second messengers such as phospholipids. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death mechanisms (Wang et al., 2001). In general, plant responses are of three kinds: maintenance of homeostasis, detoxification of harmful elements, and recovery of growth. Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. ABA signaling plays a vital role in plant stress responses as evidenced by the fact that many of the drought-inducible genes studied to date are also induced by ABA. ABA levels rise within one hour of an imposition of water stress (Bensen et al., 1988) and salt stress (He and Cramer, 1996).

Compatible solutes, or osmolytes, accumulate in organisms in response to osmotic stress. The primary function of the compatible solutes is to maintain cell turgor and thus the driving gradient for water uptake. Recent studies indicate that compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins (Hare et al., 1998; McNeil et al., 1999; Diamant et al., 2001). The compatible solutes generally found in higher plants are low molecular weight sugars (Garcia et al., 1997; Goddijn and van Dun, 1999), organic acids, polyols (Smith and Phillips, 1980; Abebe et al., 2003), and nitrogen containing compounds such as amino acids, amides such as glutamine and asparagine, imino acids, ectoine, proteins and quaternary ammonium compounds (Rabe, 1990; Rhodes and Hanson, 1993; Pareek et al., 1997; Mansour, 2000;

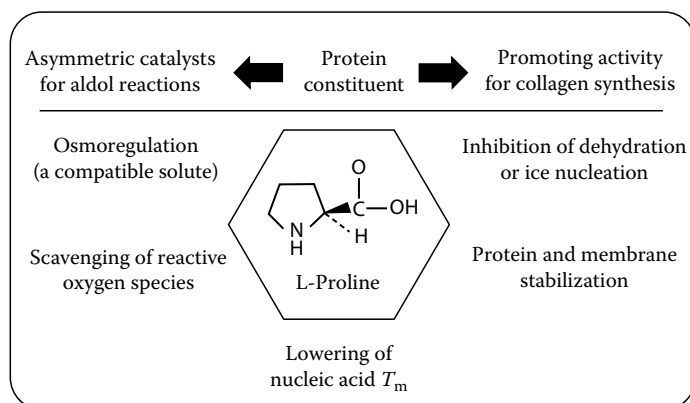
Carillo et al., 2008). Overexpression of compatible solutes in transgenic plants can result in improved stress tolerance (Sakamoto and Murata, 2002; Mattana et al., 2005; Pasquali et al., 2008).

### 9.2.2 ROLE OF PROLINE IN WATER- AND SALT-STRESSED PLANTS

Although its role in plant osmotolerance remains controversial, proline is thought to contribute to osmotic adjustment, detoxification of ROS, and protection of membrane integrity. Proline accumulation is believed to play adaptive roles in plant stress tolerance and has been proposed to act as a compatible osmolyte and to be a way to store carbon and nitrogen. Salinity and drought are known to induce oxidative stress. Early in vitro studies showed that proline can be a ROS scavenger. Proline has also been proposed to function as molecular chaperone stabilizing the structure of proteins, and proline accumulation can provide a way to buffer cytosolic pH and to balance cell redox status. Finally proline accumulation may be part of the stress signal influencing adaptive responses.

Proline may act as a component of signal transduction pathways that regulate stress responsive genes in addition to its previously described osmoprotective roles, thereby improving the tolerance to salt stress. Proline has been thought to have an adaptive role in mediating osmotic adjustment and protecting subcellular structure in stressed plants. Synthesis, degradation, and transport of proline cooperatively control its endogenous level in higher plants in response to environmental conditions. The physiological functions of proline are shown in Figure 9.1.

In *Pancreaticum maritimum* L., salt stress resulted in a decrease in the amount of ubiquitin conjugates, (a small protein targeting damaged proteins for degradation via the proteasome), particularly in the roots, and this effect was reversed by exogenous proline (Khedr, 2003). Severe salt stress resulted in an inhibition of the antioxidative enzymes, catalase and peroxidase, as revealed by spectrophotometric assays and activity gels, but the activity of these enzymes was also maintained significantly higher in the presence of proline. Salt stress also upregulated several dehydrin proteins, analyzed by western blotting, even in nonstressed plants. Thus, proline could act as a component of signal transduction pathways that regulate stress responsive genes in addition to its previously described osmoprotective roles, thereby improving the tolerance to salt stress. In ice plant (*Mesembryanthemum crystallinum*), barley (*Hordeum vulgare* L.), and wheat (*Triticum aestivum* L.) proline has a bifunctional role in the acclimation to high salt stress; an osmoregulant role in the light, and a substrate for dark respiration to supply energy for compartmentation of ions into vacuole in the dark (Sanada et al., 1995). In *Cordyline fruticosa*, proline plays an osmoregulatory role, increasing in roots not only when NaCl concentration in the nutrient solution is too high but also



**FIGURE 9.1** The physiological functions of proline. Proline has been shown to exhibit multiple functions other than osmoprotection in vitro. However, the mechanism by which proline confers stress tolerance in vivo remains poorly understood. Although proline is accumulated in many bacterial and plant cells in response to osmotic stress, proline levels are not increased under various stresses in *S. cerevisiae*. (From Takagi, H., *Appl. Microbiol. Biotechnol.*, 81, 211, 2008.)

when it is too low (Plaza et al., 2009). In some cases, proline as a free radical scavenger may be more important in overcoming stress than in acting as a simple osmolyte (Hong et al., 2000). Mattioli et al. (2008) suggested that proline plays a key role in flower transition, bolting, and coinflorescence formation. The considerable increase in free proline content in the leaves of acclimated barley plants was of a minor importance for its tolerance to dehydration (Bandurska, 2001), suggesting that proline accumulation alone is not a sufficient factor in the development of leaf dehydration tolerance to severe water deficit. The role of proline in *Halimium halimifolium* and *Pistacia lentiscus*, two contrasted species of Mediterranean shrubs, seems to be more related to a protective action in cases of severe stress conditions rather than as an osmotic agent with an osmotic potential depressing activity (Ain-Lhout et al., 2001). In *Sesbania sesban*, proline fully alleviated the salt stress induced enhancement of ribulose-1, 5-bisphosphate oxygenase activity, suggesting that proline accumulation in plants under stress must be assisting plants in maintaining the photosynthetic efficiency and the overall productivity (Sivakumar et al., 2000). Proline synthesis and its subsequent accumulation in aquatic plants is a consequence of intracellular ionic adjustments that take place under salt stress to keep the metabolic activities going on (Rout and Shaw, 1998).

### 9.3 PROLINE METABOLISM

Proline metabolism is a typical mechanism of the biochemical adaptation in living organisms subjected to stress conditions. Mitochondria have an important role regarding proline homeostasis during water stress. In many plants, proline accumulates in response to water stress and rapidly disappears upon recovery, due to variations in its cytosolic synthesis and mitochondrial degradation rates (Kiyosue et al., 1996). Moreover, since proline synthesis from glutamate requires two molecules of cytosolic NADPH, and its reverse oxidative degradation in mitochondria generates FADH<sub>2</sub> and NADH, it has been suggested that glutamate–proline cycling between cytosol and mitochondria could have a major role in redox homeostasis and metabolism (Hare and Cress, 1997). Proline metabolism is regulated by feedback inhibition mechanism (Hare and Cress, 1997; Hong et al., 2000). The synthesis of proline may have an additional effect. Intermediates in proline biosynthesis and catabolism such as glutamine and  $\Delta^1$ -pyrroline-5-carboxylic acid (P5C) can increase the expression of several osmotically regulated genes in rice (*Oryza sativa* L.), including *salT* and *dhn4* (Iyer and Caplan, 1998). Proline as a free radical scavenger may be more important in overcoming stress than in acting as a simple osmolyte.

#### 9.3.1 PROLINE SYNTHESIS

The pathway of proline biosynthesis was first elucidated in *Escherichia coli* in 1952, but only confirmed in 1987 (Leisinger, 1987). Proline biosynthesis is initiated with the ATP-dependent phosphorylation of glutamic acid by the  $\gamma$ -glutamyl kinase ( $\gamma$ -GK), encoded by the *proB* gene. The product of  $\gamma$ -GK is reduced to glutamic-semialdehyde (GSA) by the  $\gamma$ -glutamyl phosphate reductase ( $\gamma$ -GPR) encoded by the *proA* gene (Turchetto-Zolet et al., 2009). GSA cyclizes spontaneously to form P5C, which is finally reduced to proline by P5C reductase (P5CR, encoded by the *proC* gene). The proline biosynthetic route in plants resembles the bacterial pathway and uses either glutamic acid (Stewart, 1981) or ornithine (Mestichelli et al., 1979) as substrates. In plants, proline synthesis is regulated by end-product inhibition (Noguchi et al., 1968; Boggess et al., 1976). Under normal physiological conditions, both pathways contribute to proline synthesis in the cytosol. Under stressful conditions, proline is synthesized preferentially from glutamic acid (Delauney and Verma, 1993) via two intermediates: GSA and P5C. In common reed plants, free proline content and pyrroline-5-carboxylate synthetase (P5CS) activity increased with salt stress treatment, while ornithine aminotransferase (OAT) activity appeared to be unaffected, suggesting that the glutamate pathway, rather than the ornithine pathway, plays a vital role in proline accumulation during osmotic regulation (Zhen and Ma, 2009). Proline synthesis in plants can be manipulated by eliminating feedback regulation of the key regulatory enzyme of the pathway, P5CS.

### 9.3.2 PROLINE CATABOLISM

Proline accumulation and catabolism play significant roles in adaptation to a variety of plant stresses including osmotic stress, drought, temperature, freezing, UV irradiation, heavy metals, and pathogen infection. The ability of plants to degrade proline through an oxidation process has been shown clearly (Stewart, 1981). Accumulated proline is rapidly oxidized back to glutamate when osmotic stress is relieved. The first step of this process is catalyzed by a mitochondrial proline dehydrogenase (oxidase) (Sweetlove et al., 2007). Studies in *Arabidopsis* have shown rapid transcript response of proline oxidase to changing osmotic pressure, suggesting that it is a regulatory link in proline homeostasis in plants. The subsequent step in this pathway is catalyzed by delta1-pyrroline-5-carboxylate dehydrogenase. The expression of this mitochondrial enzyme is regulated by osmotic stress in *Arabidopsis* (Deuschle et al., 2001) and has recently been shown that it can act in tandem with the stress-related gene transcript to generate two types of small interfering RNAs (siRNAs) which participate in a complex manner to regulate salt tolerance (Borsani et al., 2005). The occurrence of two separate translocators for proline in the mitochondria, namely a carrier solely for proline and a proline/glutamate antiporter en route to its catabolism was shown in durum wheat (Di Martino et al., 2006). Proline (accumulated/synthesized in the cytosol) enters mitochondria via its own uniporter; inside the mitochondria Pro catabolism takes place at a rate exceeding that of the transport, as shown by the lack of accumulation of proline in the matrix; Glu is then exported outside mitochondria in exchange for further Pro via the Pro/Glu antiporter. The catabolism of proline in the mitochondria has been suggested to play multiple roles in plant physiology. It can raise the energetic status of the cell following the relief of stress conditions in addition to acting as an available reservoir of carbon and nitrogen (Hare and Cress, 1997). Recently, association between proline and programmed cell death has been reported with proline-treated plant cells undergoing a necrotic response with features consistent with the production of ROS and programmed cell death (Deuschle et al., 2004). It has been suggested to be important in the protection of catalase, peroxidase, and Complex II activities (Hamilton and Heckathorn, 2001; Ozturk and Demir, 2002). In all instances, it would appear likely that the mitochondrially localized enzymes responsible for the degradation of proline play a prominent regulatory role in these processes. The activity of the proline degrading enzyme, proline dehydrogenase (PDH), decreased under salt stress in common reed plants, indicating that proline catabolism may be responsible for proline accumulation in response to salt stress (Zhen and Ma, 2009).

### 9.3.3 ENDPRODUCT INHIBITION

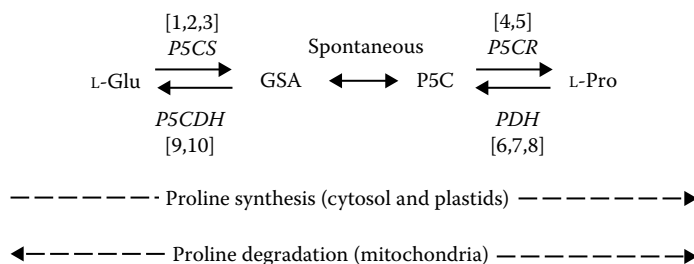
Proline manifests a conspicuous ability to control its own biosynthesis. Exogenous application to plant tissues of an amount of proline sufficient to increase the endogenous pools enhanced the rate of proline oxidation as a result of a feedback inhibition process (Stewart, 1972; Boggess et al., 1976). It is known that proline oxidase, one of the enzymes involved in proline degradation, can be induced by high concentrations of proline (Boggess et al., 1976; Adams and Frank, 1980). Feedback inhibition of proline synthesis does not occur under water-stress conditions. In sorghum and chickpea, feedback inhibition of P5CS by proline during moisture stress was observed (Dalvi et al., 2007). In bacteria, proline biosynthesis has been shown to be regulated by the end-product inhibition of  $\gamma$ -GK activity (Smith et al., 1984). A similar situation was also observed in plants. The  $\gamma$ -GK activity of Vigna P5CS was inhibited by proline, but its GSA dehydrogenase activity was not affected, suggesting that the  $\gamma$ -GK is the rate-limiting step in proline biosynthesis in plants (Zhang et al., 1995). The P5CS is the rate-limiting enzyme in proline (Pro) biosynthesis in plants which is encoded by two highly homologous genes in *Arabidopsis* and many other plants, and is subject to feedback inhibition by proline (Yoshiba et al., 1997; Fujita et al., 1998; Ginzberg et al., 1998). It is controlled at the level of P5CS transcription and through feedback inhibition of P5CS by proline (Zhang et al., 1995; Hong et al., 2000).

### 9.3.4 GENETIC MANIPULATIONS, OVEREXPRESSION OF GENES, AND TRANSCRIPTIONAL REGULATION OF GENES INVOLVED IN PROLINE SYNTHESIS

In plants, many key enzymes of metabolic pathways are generally encoded by redundant genes, which may be generated by gene duplication events during the evolutionary history of the organisms. Gene duplication produces two functionally identical copies that act in a totally redundant way immediately following the duplication event, and are often followed by sequence alterations causing changes in transcriptional regulation and contributing to evolution of functional divergence. The genetic manipulations of proline metabolic pathways in plants are summarized in Figure 9.2.

Effects of genetic manipulation of proline synthesis can be plant species specific. Overexpressing soybean *P5CR* gene in transgenic tobacco did not increase osmotolerance (Szoke et al., 1992). The overexpression of the *P5CS* encoding gene in transgenic tobacco plants resulted in increased proline production and conferred tolerance of these plants to osmotic stress, confirming that *P5CS* is of key importance for the biosynthesis of proline in plants (Kishor et al., 1995). Field trials in South Africa with *P5CR* transgenic soybean lines supported improved drought performance and higher heat tolerance compared to wild type cultivars (de Ronde et al., 2004). However, mutants displaying higher proline accumulation can also be salt hypersensitive (Lui and Zhu, 1997).

It has been shown that transcriptional control of the *P5CS* gene is important for the regulation of accumulation of proline during osmotic stress in plants. Studies of transcriptional regulation of genes involved in proline synthesis confirmed developmental regulation. In young *Arabidopsis* plants, beta-glucuronidase (GUS) analysis of *AtP5R* (*Arabidopsis P5CR* gene) promoter revealed high expression in apical meristem and young leaf, in root meristem, secondary root primordia and root vascular cylinder. In young leaf high *P5R* expression could be detected all over the leaf blade,



**FIGURE 9.2** Genetic manipulations of proline metabolic pathways in plants. The main precursor of proline synthesis is L-glutamic acid (L-Glu). L-Glu is first reduced to glutamate semialdehyde, which spontaneously cyclizes to pyrroline-5-carboxylate (P5C), by P5C synthase (P5CS). The second reduction, of P5C to proline, is catalyzed by P5C reductase (P5CR). This pathway is found in the cytosol and in plastids. Proline is catabolized to Glu in mitochondria by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). Examples of genetic manipulations of proline metabolic pathway and impact on osmotolerance: [1] Overexpression of mothbean *P5CS* in tobacco plants. Those transgenics were better salt tolerant (Kishor et al., 1995); [2] Overexpression of *P5CS* in antisense orientation in *Arabidopsis* (Nanjo et al., 1999a) and [3] *p5cs1* insertional mutation (Székely et al., 2008) resulted in reduced osmotolerance; [4] Overexpression of soybean *P5CR* gene in tobacco (Szoke et al., 1992) did not modify osmotolerance; [5] Overexpression of *Arabidopsis P5CR* (*AtP5R*) gene in soybean improved drought and heat stress (de Ronde et al., 2000, 2004); [6] Overexpression of *PDH* did not change osmotolerance in *Arabidopsis*, except in the presence of exogenously supplied Pro. In these conditions *PDH*-sense lines were better osmotolerant than WT. [7] Higher salt tolerance was observed in *Arabidopsis* plants overexpressing *PDH* in antisense orientation by Nanjo et al. (1999b), but not by Mani et al. (2002). Study of modifications of Pro catabolism in *Arabidopsis* in relation to P5/GSA and/or proline toxicity: [6] Overexpression of *PDH* decreased sensitivity to externally supplied proline; [7] decrease of *PDH* activity by antisense strategy (Mani et al., 2002); or [8] knock-out mutation (Nanjo et al., 2003) increased sensitivity to Pro; [9] *P5CDH* overexpression decreased sensitivity to externally supplied proline (Deuschle et al., 2004); [10] *p5cdh* knock-out mutants were hypersensitive to proline (Deuschle et al., 2004) (From Verbruggen, N. and Hermans, C., *Amino Acids*, 35, 753, 2008.)

while in old leaves, expression was restricted to the veins, hydathodes, guard cells and base of trichomes (Hua et al., 1997). In flowering plants, high *AtP5R* expression could be detected in rapidly dividing cells, such as root meristem, and cells or tissues undergoing changes in water potential, such as hydathode, guard cell, ovule, developing seed, and pollen grain (Hua et al., 1997). *AtHAL3a*, an *HAL3* homolog gene in *Arabidopsis thaliana* overexpressing transgenic plants exhibit improved salt and sorbitol tolerance (Ikuko et al., 2004). Overexpression of *NtHAL3a* improved salt, osmotic, and lithium tolerance in cultured tobacco cells. Proline degradation can also be manipulated and depends on prior proline transport to mitochondria. Overexpression of *PDH* in *A. thaliana* did not result in morphological abnormalities, probably because proline homeostasis relies on regulated transport between cell compartments (Nanjo et al., 1999; Mani et al., 2002).

### 9.3.5 IMPACT OF ENVIRONMENTAL STRESSES ON PROLINE

Proline accumulation is a common physiological response in many plants in response to a wide range of biotic and abiotic stresses. Extensive research in this area has revealed proline accumulation as a universal response of plants under stress. Proline accumulation in dehydrated plants is generally due to the concerted activation of proline biosynthesis in the cytosol and inhibition of its catabolism in the mitochondria (Kiyosue et al., 1996). Proline accumulation due to water or salt stress results from a stimulated synthesis in the tissue, an inhibited oxidation, or an impaired incorporation of proline into proteins. Clearly, under such conditions, the mechanisms of feedback inhibition cannot function. Indeed, this was proven by Boggess et al. (1976) who showed that proline does not inhibit its own synthesis in wilted barley and tobacco leaves. The increased synthesis of proline due to wilting seems to be related to the first step of this synthesis. On the other hand, water stress and salinization inhibit proline oxidation in the mitochondria and alter the permeability of the mitochondrial membrane (Miller et al., 1971; Nir et al., 1971; Sells and Koeppel, 1980). Further, the incorporation of proline into protein is inhibited by water stress, thereby also leading to proline accumulation under stress conditions (Stewart et al., 1977). The step affected in protein synthesis is probably the translation step (Hsiao, 1973); however, this process is not obligatory, because proline accumulation was observed when protein synthesis inhibitors, such as cycloheximide, were applied (Boggess and Stewart, 1980). The state of the hormonal balance in plants is suggested to play a considerable role in mediation of proline accumulation under water or salt stress (Boggess and Stewart, 1980). The key role in the osmotic adaptation of plants has been ascribed to ABA. The reduction in turgor is accepted as the primary trigger of proline accumulation in plants subjected to conditions of drought and salinity. The loss of turgor activates a complex sequence of adaptive events correlated to the level of stress, plant tolerances, and plant growth.

## 9.4 PROLINE ACCUMULATION AS AN OSMOREGULATORY RESPONSE

Proline interacts with enzymes to preserve protein structure and activity within the cell. In vitro studies have shown that high concentrations of proline reduce enzyme denaturation attributable to heat, freeze-thaw cycles, and high NaCl (Pollard and Wyn Jones, 1979; Rajendrakumar et al., 1994). Moreover, proline protects proteins and membranes from damage by inactivating hydroxyl radicals or other highly reactive chemical species that accumulate when stress inhibits electron-transfer processes (Smirnoff and Cumbes, 1989).

The accumulation of proline seems to be associated with adaptation to temperature stress. Free proline concentrations increased under both high (Ashraf et al., 1994) or low temperature (Tarnizi and Marziah, 1995; Wang and Cui, 1996), and proline could serve as a stress indicator in plants exposed to these unfavorable growth conditions. Heavy metals or herbicides are other environmental factors that induce proline accumulation in plants. *Vicia faba* plants grown in hydrocarbon-polluted soils accumulated high levels of proline (Malallah et al., 1996). High-Pb concentrations reduced sunflower plants' biomass but increased the concentration of free proline (Kastori et al., 1996).

The leaf proline content also increased in pea plants (Kamenova-Yukhimenko et al., 1995) or *Vigna radiata* (Arora and Saradhi, 1995) grown in nutrient solutions containing high-Cd concentrations. Chlormequat given to soybean plants increased the leaf proline content (El-Kheir et al., 1994). The foliar application of paraquat and diquat to *Parthenium hysterophorus* resulted in a reduction of the proline level 48 h after application followed by an increase 72 h after spraying (Kore and Patil, 1995). The foliar application of fomesan, imazaquin, metobromuron, and mettolachlor to soybean plants resulted in reduced levels of proline and some other amino acids, probably because of a disturbed protein synthesis (Stoirnenova and Stoyanova, 1995). Chlorsulfuron, norflurazon, and triallate increased the proline content in pea and *V. faba* (Fayez and Kristen, 1996). Increased levels of proline were also found in barley plants infested with aphids because of low water potentials (Fayez and Kristen, 1996). It is suggested that the changes in proline could be considered as a protective/adaptive mechanism against expected injuries resulting from pollution or herbicides.

Proline accumulation in dehydrated plant tissues was first noted in 1954 by Kemble and Mac-Pherson. Further experiments showed that the de novo synthesis of proline was involved, as the amounts were greater than those released by proteolysis. Proline accumulation was observed in many species as a result of exposure to water or salt stresses. Several reports correlate this phenomenon with stress resistance indicating that a better performance and survival can be expected in species that accumulate proline. Much evidence is available to corroborate the proline action as a compatible solute regulating and reducing water losses from dehydrated cells. The increase in the proline content during water stress is inversely proportional to the initial proline concentration in plant organs (Venekamp and Koot, 1988). It was found that the generative parts of bean plants contained considerably lower concentrations of proline than the vegetative parts after withholding water. The accumulation of proline during tissue dehydration is time dependent (Rajagopal et al., 1977). The accumulated proline is lost rapidly as a result of recovery from water stress but not as an immediate response to salt removal from the media.

All the above-mentioned points are addressed in detail in the following sections.

#### 9.4.1 OSMOTIC ADAPTATION OF BACTERIA AND ALGAE

Generally, microbes respond to water potentials by accumulating intracellular compounds that are compatible with the cellular metabolic function. There is a natural ranking or differing osmolyte preferences among species of bacteria (Csonka, 1989). The accumulation of compatible solutes in soil bacteria cells subjected to increased salinity depends either on direct uptake from the soil solution (or the rhizosphere) or on biosynthesis via the uptake of a direct precursor such as choline (Dupont et al., 2003). Both mechanisms occur simultaneously in a majority of bacteria, as in *Sinorhizobium meliloti*. *Azotobacter chroococcum*, *Azospirillum brasilense*, and *Klebsiella pneumoniae*, which are adversely affected by high osmotic strength, show osmotic adjustment when exposed to these conditions. *A. chroococcum* ZSM4 accumulates trehalose and glutamate, proline and glutamate in *A. brasilense* SHS6, and trehalose and proline in *K. pneumoniae* (Madkour et al., 1990). Glycine betaine was accumulated in all strains. This chapter focuses on betaine transport and glycine betaine biosynthesis in this bacterium, a typical representative of the rhizosphere and the microsymbiont of lucerne root nodules. Also covered are the salt tolerance of various Rhizobiaceae strains and their capacity to use choline and glycine betaine as osmoprotectants. Partial alleviation of salt stress was obtained by adding low concentrations of proline or betaine to the growth medium of *Thiobacillus ferrooxidans* (Kieft and Spence, 1988). Similar results were obtained for *Rhizobium* species (Gloux and Le Rudulier, 1989). In response to increased salt concentrations, algae also synthesize osmoregulatory solutes to counterbalance the low water potential of the growth media. Some nitrogen-fixing cyanobacteria (blue-green algae) display remarkable abilities to survive water stress, experienced either as salinity or as drought. Survival during water stress is achieved by regulation of ion fluxes for immediate osmotic adjustment to rapidly prevent turgor loss, and by a long-term adaptation plan, facilitated through selective expression of "tolerance" genes and novel

stress proteins. Such osmotic adaptation comprises of accumulation of compatible solutes conducive to metabolism and repair of oxidative damage caused by excess of free radicals. A variety of osmolytes are synthesized such as sugars, polyols, and betaines, respectively conferring low, medium, and high level of tolerance (Apte, 2001). Proline plays a key role in the osmoregulatory mechanism of *Chlorella autotrophica* (a marine microalga) when exposed to high salinities (Ahmad and Hellebust, 1984). In this alga, proline accumulation begins immediately after an osmotic shock without a lag phase. This alga is not dependent on protein synthesis and requires light, because photosynthesis supplies the required energy for proline biosynthesis.

#### 9.4.2 ACCUMULATION OF PROLINE IN CALLUS CULTURES AND ISOLATED CELLS

Compatible solutes were involved in the response of *Thellungiella holophila* and *A. thaliana* calli to salt stress (Zhao et al., 2009). Exogenous proline increases the cadmium accumulation in callus and regenerated shoots of *Solanum nigrum* (Xu et al., 2009). Endogenous free proline content in tomato calli increased gradually in response to elevating polyethylene glycol (PEG) concentrations (Shtereva et al., 2008). PEG-induced osmotic stress tolerance was found to be associated with the accumulation of free proline in 1-month-old calli of two indica rice genotypes (Ahmad et al., 2007). Proline increased significantly in date palm calli in response to salinity (Al Mansoori et al., 2007). During the induction stage, the increase in endogenous free proline content was more pronounced in the progeny of the cultivar which exhibited higher percentage of callus induction. Therefore, the better dedifferentiation process could be related to proline content, which adjusts the intracellular osmotic pressure between the cytoplasm and the vacuole.

Callus cultures of rice, adapted to grow under increasing levels of NaCl, accumulated considerable amounts of free proline compared with unadapted cells (Kishor, 1988). This trait was not lost when the cells were transferred through 10 passages in the absence of selection pressure and regrown on salt. On the other hand, mature embryo-derived calli from rice cultivars differing in their salt tolerance also accumulated proline when exposed to NaCl, KCl, or mannitol (Lutis et al., 1996). This accumulation did not depend on the nature of the stressing agent or the stress intensity and did not appear to be involved in osmotic adjustment; therefore, it was considered to be a symptom or injury in the stressed rice calli and not an indicator or resistance. In an embryogenic callus culture of lemon, selected for resistance to salinity, the proline concentration significantly increased as compared with control cells (Piqueras et al., 1996). The transfer of salt-tolerant cell cultures of alfalfa to NaCl-containing medium resulted in a tenfold increase in proline concentrations (Petrusa and Winicov, 1997). In *Brassica napus*, both unselected and tolerant calli responded to water stress by osmotic adjustment and proline concentration (Chandler and Thorpe, 1987). Increases in proline concentrations were approximately linear in tolerant calli, reaching a maximum of 175 dry weight and 520  $\mu$ M in selected calli. This accumulation was correlated with growth inhibition and negatively correlated with the culture age for tolerant calli. Callus cultures of *Medicago sativa* accumulated proline in response to NaCl (Shah et al., 1990). This accumulation was enhanced by calcium and was positively correlated with salt tolerance. The salt tolerance of sugar beet calli was also accompanied by a significant increase in the proline concentration under conditions of high salinity (Le Dily, 1991). The proline content in a callus culture of pearl millet grown in 1% NaCl increased more than 20-fold compared with nonsalinized controls (Das et al., 1990). Exposure of tobacco callus cultures to osmotic shock greatly enhanced the proline accumulation in proportion to the amount of absorbed sorbitol (Eberhardt and Wegmann, 1989). NaCl-resistant cell lines of tobacco increased the proline content within 5–10h after transplantation to a selective medium. In the wild strain, the proline content remained unchanged over a 24h period (Watad et al., 1983; Kiryan and Shevyakova, 1984). A correlation between the viability of cells in a saline environment and the proline content was observed. The intracellular proline concentration was positively correlated with the osmotic potential indicating that proline was a component of the osmotic adjustment of the cells (Binzel et al., 1987). Glutamate was the main source of the newly produced proline, and the relative



contribution of the catabolic pathway was small. Cell lines selected for resistance to salt stress responded to water stress by accumulating markedly more proline than the wild type (Watah et al., 1983). This response was stable through at least eight generations and was fully reversible. Similar results were obtained with cultured cells of a salt marsh grass (*Distichlis spicata* L.) (Ketchum et al., 1991). Most of the accumulated proline effective in osmoregulation was found in the cytoplasm. Therefore, it plays a major role in the osmotic adjustment of the vacuole. Cells maintained a cytoplasmic proline concentration at least one order of magnitude greater than that of the vacuole. Proline accumulation was inhibited by cycloheximide but not by actinomycin D. This indicated that mRNA translation, not mRNA transcription, is required before proline production. Reports are also available on a preferential accumulation of proline in nontolerant cells, as opposed to tolerant cells, indicating a dependence on a salinity threshold (Jain et al., 1987).

A significantly higher level of free proline content was observed in sugarcane calli grown under salt stress (Patade et al., 2008). An inverse correlation was found between the salt-tolerance and NaCl-induced proline accumulation in intact plants and cell cultures of *Salicornia europaea* L., *Chenopodium album* L. and in two cotton (*Gossypium hirsutum* L.) cultivars, although the accumulation of proline in leaves was of no importance for adaptation or survival of the plants (Shevyakova et al., 1998).

Cultured cells of sorghum exposed to water stress by the addition of PEG to the growing media increased proline content significantly (Bhaskaran et al., 1985). The magnitude of this increase was not correlated with the drought tolerance of the individual varieties ruling out the role of proline as an osmoprotectant in sorghum, as well as in other cereal crops.

#### 9.4.3 SEED GERMINATION AND PROLINE CONTENT UNDER STRESS

Most of the research on proline as an osmoregulatory compound has been carried out on the vegetative parts of plants. Little attention has been paid to the reproductive organs, especially seeds. Information on the osmotic adjustment of seeds under stress conditions is available. Drought-induced accumulation of soluble sugars, soluble proteins, free amino acids, and proline in seeds of *V. faba* L. cultivars was seen when exposed to different levels of PEG (Al Tayeb, 2006). Proline was used as an indicator of temperature stress in bean seeds (Machado et al., 2004). Proline content was used to predict the rate of deterioration in corn seed that were treated with ascorbic acid and germinated on NaCl stress condition (Anwar and Widajati, 2002). A close relationship between proline content and quality of the corn seed was found; the higher the proline content of the seeds, the lower the seed quality. Although there was a significant effect of salt stress on proline contents of all the different parts of germinating seeds of spring wheat cultivars, there was no consistent pattern of proline accumulation with time (Riaz, 2001). Salinity led to an accumulation of proline and total soluble sugar in the radicals of the sensitive genotype of *Cicer arietinum* (Soussi et al., 2001). Contents of proline, saccharides, and soluble proteins decreased in the germinating seeds during 3-d osmotic and salt stress of mung bean (*V. radiata*) (Zayed and Zeid, 1998). An approximately fourfold increase in free proline was observed prior to radical emergence in *A. thaliana* seeds (Hare et al., 2003). A dose-dependent inhibition of *Arabidopsis* seed germination by millimolar concentrations of proline capable of feedback inhibition of proline synthesis reinforced the role of proline synthesis in promoting germination. The ability of the artificial oxidants methylene blue and phenazine ethosulphate to overcome the inhibitory effects of proline suggests a functional link between elevated proline synthesis and increased oxidative pentose phosphate pathway activity and the importance of coupling of both pathways in stimulating germination. During germination of peanut seeds subjected to NaCl salinity stress, proline, and glycine–betaine concentrations in the embryonic axis increased continuously (Girija et al., 2002). Two enzymes play an important role in controlling the level of proline. Proline oxidase catalyzes the conversion of proline to glutamate, thus reducing the concentration of proline. Another enzyme, gamma-glutamyl kinase, plays an important role in the synthesis of proline. Addition of calcium chloride to NaCl-stressed peanut

seedlings lowered the proline concentration by increasing the level of proline oxidase and decreasing gamma-glutamyl kinase activities. Salinity stress, in the absence of calcium, increased proline due to reduced proline oxidase activity and increased gamma-glutamyl kinase activity both in the cotyledons and embryonic axis of peanut seedlings. Thus calcium ions increase glycine–betaine production but decrease proline levels in NaCl stressed peanut seedlings. Water absorption and germination rate of the seeds were negatively correlated with the salt stress (Zhi and Li, 2009). The contents of soluble sugar, soluble protein, and proline of the seedlings were higher in high salt stress than that in low salt stress. Germination rates of seeds of *Glycine max* and *Phaseolus vulgaris* were affected by exposure to NaCl, with critical thresholds at 9 and 12 g/L, respectively, while for *Mucuna poggei*, *Vigna unguiculata*, and *Phaseolus adenanthus*, critical thresholds were more than 21 g/L (Taffouo et al., 2008). Germination rate of *Pennisetum* Rich. was decreased significantly with increasing contents of PEG, but the radical/embryonic bud was increased (Jiao et al., 2009). Drought resistance in annual medics (*Medicago* spp.) plants is gained by their capability of proline accumulation (Ghamari-Zare et al., 2009). Seed germination of *Dianthus japonicus* was suppressed at over 200 mM NaCl and proline content was increased in adversely affected plants (Heo et al., 2007). Proline substantially accumulated in the seedlings of wheat cultivar (Doha) exposed to various levels of salt stress (Yasseen et al., 2006). There was a linear relationship between accumulation and the increase in NaCl concentration. Salt concentration of 50–100 mM increased seed germination and growth of *Elaeagnus angustifolia*, while at 300 mM, it was reduced by 50% (Yunus et al., 2006). Proline content was insignificantly changed at low salinity but remarkably increased at severe stress. There was strong negative correlation between germination capacity and increase in proline content in germinating embryos of sorghum (Thakur and Sharma, 2005). Germination percentage decreased in cultivars of sugarcane due to salinity, while proline content increased by two- to threefold over the controls (Vasanth and Rao, 2005). Increased antioxidant enzyme activity coincided with enhanced proline content in *Cassia angustifolia* (Agarwal and Pandey, 2004). Increase of membrane permeability of *Hedysarum scoparium*, *H. fruticosum* var. *laeve*, *Caragana korshinskii* and *Onobrychis viciifolia* seedlings caused by salt stress was negatively correlated with salt concentrations, while the cumulative amount of proline had no direct correlation with salt tolerance (An et al., 1995). Time at which proline accumulation peaked indicated the sensitivity of plants to salt. A decrease in germination percentage, root length, shoot length, and fresh mass of *Phaseolus mungo* was noticed with an increase in NaCl concentration (Dash and Panda, 2001). Salt stress increased proline accumulation in the cotyledons and roots of germinating groundnut seeds (Satakopan and Rajendran, 1989). Proline accumulated in the endosperm and radicals of germinating barley seeds with increasing NaCl concentrations in the growing media (Yasseen et al., 1989). This proline probably originated from the degradation of stored protein in the endosperm. Under increasing levels of salinity, germinating seeds from salt-tolerant cultivars of rice contained higher levels of free amino acids than salt-sensitive cultivars (Dubey and Rani, 1989). In contrast, irrigation of Cajeme wheat with saline water resulted in a continuous decrease in free proline in the grains (Devitt et al., 1987). This decrease was associated with increased protein concentrations suggesting either the rate of protein incorporation was accelerated under salinity or that free proline accumulation was stunted in other parts of the plant.

#### 9.4.4 OSMOTIC ADJUSTMENT IN HALOPHYTES

Halophytes are plants capable of growing and reproducing in highly saline environments. These plants usually absorb large amounts of NaCl, which is believed to be sequestered in the cell vacuoles; otherwise, enzyme activity would be impaired. Although halophytes are naturally adapted to salinity, their salt-tolerance limits are greatly influenced by their provenance and developmental stage. Halophytes are characterized by considerable asymmetry in the distribution of osmotica within their cells, particularly between the vacuole and the cytoplasm (Yeo, 1981). The tolerance of all halophytes to salinity relies on controlled uptake and compartmentalization of Na<sup>+</sup>, K<sup>+</sup>, and

Cl<sup>-</sup> and the synthesis of organic compatible solutes, even where salt glands are operative (Flowers and Colmer, 2008). Proline, known to be accumulated in the cell cytoplasm to balance the osmotic potential of the accumulated salt in the vacuole, does not play a role in the osmotic regulation of halophytes. For example, in halophytic Chenopodiaceae, such as *Suaeda monoica*, *Atriplex spongiosa* and *Arthrocnemum fruticosum*, proline accumulation was observed only at high inhibitory salinities, promoting normal growth (Doddema et al., 1986; Storey and Wyn Jones, 1997). Similar results were obtained with the halophyte *M. crystallinum* growth at 400 mM NaCl (Demming and Winter, 1986). Proline accumulation preceded the shift of crassulacean acid metabolism (CAM) in these plants, but only under light (Sanada et al., 1995). The results suggested that in *Mesembryanthemum*, proline has a bifunctional role in the acclimation to high salt stress: an osmoregulatory role in the light and as a substrate for dark respiration to supply energy for compartmentation of ions into vacuoles in the dark. Proline concentration in *Spartina* varied with N availability, although the higher accumulation could not alleviate the growth inhibition caused by the high salinity level (van Diggelen et al., 1986). *Sporobolus virginicus* showed high salt tolerance due to its ability to accumulate high sodium and the secretion of salt through leaves for osmotic adjustment to reduce the osmotic potential of the cell to avoid dehydration and death (Somsri and Arunee, 1994). All accessions of *Atriplex canescens* responded to salinity by increasing their uptake of Na, which is the primary mechanism of osmotic adjustment to salinity in this species (Glenn et al., 1996). It is suggested that differences in tendency to accumulate Na or K among *A. canescens* genotypes are related to their specialization for saline or xeric habitats, respectively. Exposure of *Saticornia europaea* to short-term osmotic shock resulted in a rapid increase in free Mg<sup>2+</sup>, followed by a five-fold increase in sugar concentration, either of which could have provided adequate osmoticum to prevent excessive water stress if mostly confined to the cytoplasm (McNulty, 1985). Subsequent adjustment can only be explained by a reapportionment of compatible osmotica within the cells. Such reapportionment would permit the osmotic adjustment in halophytes to occur primarily by the absorption of extraneous ions. *Salvadora persica*, an evergreen perennial halophyte capable of growing under extreme conditions, accumulates Na<sup>+</sup> as a primary osmoticum (Maggio et al., 2000). The concentration of Na<sup>+</sup> in leaves of salinized plants was approximately 40-fold greater than that measured in nonsalinized controls, and this was associated with significant reductions in leaf K<sup>+</sup> and Ca<sup>2+</sup> concentrations. In addition, a significant accumulation of proline, probably associated with osmotic adjustment and protection of membrane stability, occurred in roots of salinized plants. The salt tolerance of the annual halophyte *Cakile maritima* depends on the growth stage and severity of the salt stress (Megdiche et al., 2007). *C. maritima* plants, subjected to a high salt stress (particularly at 400 mM NaCl) accumulated high concentrations of proline and total soluble carbohydrates.

#### 9.4.5 NEGATIVE RESPONSE

Despite the abundance of reports on proline accumulation in plants growing under drought or salinity, a few others demonstrate a negative response as far as osmoregulation is concerned. For example, the contribution of proline to osmotic adjustment in legumes is of minor importance (Larher et al., 1987). No significant accumulation of proline in the leaves of green gram seedlings (Garg, 1987) or maize plants (Garg and Garg, 1982) was observed when grown in sodium bicarbonate or sodium carbonate salts. Increased concentrations of chloride or sulfate salts or PEG could not effectively stimulate proline accumulation in sugar cane leaves of a salt-sensitive variety (Naik and Joshi, 1983). Similarly, pigeon pea plants failed to accumulate proline at high-salinity level (Joshi, 1984). The lack of this adaptive mechanism may explain the failure to develop salt tolerance in cultivated pigeon pea or sugar cane. In a more recent work, however, salt tolerance could be found in wild relatives of pigeon pea belonging to the genera *Atylosia*, *Dunbaria*, and *Rynchosia* but without correlation between salinity tolerance and proline accumulation (Subbarao et al., 1990). In *Andropogon glomeratus*, a C<sub>4</sub> nonhalophytic salt marsh grass, proline played no role in osmotic adjustment, since very high levels of salinity are required to

increase its concentrations (Bowman, 1988). One of the adaptive mechanisms suggested as being associated with osmotic adjustment is a restriction in cell expansion (van Volkenburgh and Boyer, 1985). Extensin, a major plant cell wall glycoprotein in dicots, was found to be a hydroxyproline-rich glycoprotein (Cassab and Vamer, 1988). Therefore, some work was carried out to determine changes in cell wall protein induced by salt stress and expressed as changes in proline and hydroxyproline concentrations. No significant effect of stress on the proline and hydroxyproline contents was found in a purified cell wall fraction of sunflower (Golan-Goldhirsh et al., 1990). Therefore, changes in the physicochemical properties of the cell wall accompanying osmotic adjustment appear to lie in other posttranslational modifications of extension. Cell membrane stability of four *Arachis* cultivars exposed to PEG was tested in response to drought tolerance (Deb et al., 1996). It was found that proline was not effective in controlling the physiological status of the cell membrane and its stability. Water-stressed pea seedlings did not significantly accumulate proline (Generozova et al., 2009). Significant negative correlations were observed in barley genotypes between  $\text{Na}^+$ -induced  $\text{K}^+$  efflux (an indicator of salt tolerance) and leaf glycine–betaine and proline (Chen et al., 2007), suggesting that hyperaccumulation of known major compatible solutes in barley does not appear to play a major role in salt-tolerance, but rather, may be a symptom of salt-susceptibility. The small (twofold) increase of proline content in salinity stressed plants indicates proline does not play a significant role in olive stress response (Rejskova et al., 2007). Other reports are also cautioning its use (Lin and Kao, 2001; Hare et al., 2002; Heuer, 2003). Different plant species, proline concentrations, and treatment stages may explain the discrepancy between the effect of exogenous application of proline and its negative effects (Ashraf and Foolad, 2007).

## 9.5 EXOGENOUS PROLINE APPLICATION

Plants growing in saline environments usually accumulate large amounts of NaCl in their tissue. Because Na and Cl are inhibitory to a large number of enzymes, their presence in the cytoplasm should be minimal. Evidence of the compartmentation of electrolytes between the cytosol and the vacuole is available. The necessary osmotic balance between the two compartments is achieved through the accumulation of organic solutes in the cytoplasm. Proline is one of these compatible solutes. Besides playing an osmotic role, it should protect enzymes against denaturation or inhibition of activity. This could be determined easily by adding exogenous proline to the assay media or to crude extracts. Contradictory reports for differing plant species are known. For example, full protection of phosphoenolpyruvate (PEP) carboxylase against NaCl inhibition was obtained in two Poaceae species with a proline concentration between 200–800 mM, and proline behaved as a competitive inhibitor in Chenopodiaceae (Manetas et al., 1986). Similar results were obtained from the activities of NAD-malate dehydrogenase, glucose-6-phosphate dehydrogenase, NADP-isocitrate dehydrogenase, and glyceraldehyde phosphate dehydrogenase (Schwab and Gaff, 1990). Almost full protection of enzyme activities was obtained when proline was added at a molar ratio of 2:1 (protectant to salt) simultaneously with the addition of salt to the reaction media. No protection was found when proline was added after 1 h preincubation of enzyme extracts with high salt concentration. Extracts from air-dried leaves recovered almost fully after more than 5 h of preincubation in 1 M proline. Exogenous proline supplied to radish seedlings reduced tissue Hg levels owing to the inhibition of Hg uptake (Khanna et al., 1995).

One approach to assess the metabolic consequences of proline accumulation in response to stress is to examine its exogenous application to whole organism or tissues. Exogenously applied proline stimulated the growth of bacteria subjected to osmotic stress (Britten and McClure, 1962; le Redulier and Valentine, 1982). Proline has also been applied to higher plants to determine its ability to counteract the inhibitory effects of environmental stress, mainly water or salt stress. The exogenous application of proline has been suggested to be an effective approach in improving crop salt tolerance in groundnut (Jain et al., 2001), melon (Kaya et al., 2007), and tobacco (Hoque et al., 2007). The addition of 100 mM proline to a Hoagland solution containing 120 mM NaCl neutralized

the effect of salinity on pea plants (Bar-Nun and Poljakoff-Mayber, 1977). Incubation of *Commelina communis* epidermal tissue in proline inhibited stomatal opening (Klein and Itai, 1989). The addition of 10 mM proline to cultured barley embryos increased shoot elongation under saline conditions (Lone et al., 1987). This effect was attributed to the ability of proline to decrease the leaf salt load. Proline allowed an enhanced K/Na discrimination in transport to the shoots and a better salt exclusion from the shoots with retention in the roots. The callus lines of *C. arietinum* grown in a medium containing 100 mM NaCl and 10 mM proline increased their fresh and dry weights (Pandey and Ganapathy, 1985). Optimal concentrations of proline increased the cellular levels of K and decreased Na and Cl levels. Spraying cotton plants grown under conditions of low soil water potential with proline solutions counteracted the effects of stress, especially at moderate and high stresses (Gadallah, 1995).

Exogenous proline had no effect on the germination of tomato seeds under water or salt stress (Wu, 1987) or on the ribosome stability in the presence of 250 or 500 mM KCl (Brady et al., 1984), but it increased pollen germination when exposed to brief temperature stress (Thind and Malik, 1994). The presence of 1 or 10 mM proline in media containing 100 or 200 mM NaCl had little effect on the growth of the salt-adapted callus of rice (Kishor, 1989). Some concentrations significantly increased the growth of salt-unadapted callus. Exogenous proline has been reported to protect plants under stress. It improved the tolerance of somatic embryos of celery (*Apium graveolens* L. cv. SB 12) to partial dehydration (Saranga et al., 1992). Okuma et al. (2000) found that exogenous proline improved the growth of salt-stressed tobacco cell cultures and the improvement was attributed to the role of proline as an osmoprotectant for enzymes and membranes against salt inhibition rather than as a compatible solute. Exogenous proline partly alleviated growth of *Pancreaticum* seedlings reduced by salt stress (Khedr et al., 2003). Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *P. maritimum* L. to salt stress. The ability of exogenous proline to maintain higher water content in severely stressed seedlings might be attributed to its contribution to osmotic adjustment both directly by increasing the internal proline content and indirectly by increasing the internal contents of other amino acids. Exogenous proline increased the protein content of the shoot and root at all levels of salt stress. Changes in growth and protein contents as a result of exogenous proline correlated with increases in the internal content of proline suggesting that proline was taken up into the roots and transported to the shoots. Ion fluxes across the plasma membrane may be regulated by low concentrations of proline in barley roots (Cuin and Shabala, 2005).

On the other hand, despite its protective role under a variety of stress conditions, external supply of proline was also found to be toxic to plant and animal cells (Hellman et al., 2000; Heuer, 2003). High concentrations of proline (50 and 100 mM) inhibited the growth of NaCl-stressed as well as NaCl-nonstressed callus cultures of mung bean (Kumar and Sharma, 1989). Proline (10 mM) inhibited the growth of salt grass suspension cultures in the presence of 260 mM NaCl (Rodriguez and Heyser, 1988). Exogenous [<sup>13</sup>C] proline inhibited the normal biosynthesis of proline that would have occurred in suspensions grown at this salinity level. It has been proposed that proline-induced damage is mediated by P5C/GSA, which, if not metabolized rapidly, might induce cell death. Exogenous proline was found to reduce the growth of roots of rice seedlings and the amount of inhibition increased with increasing the concentration of proline from 1 to 4 mM. This inhibition was attributed to the observation that proline-induced cell wall bound peroxidases which resulted in increased lignin synthesis, thereby stiffening the cell wall and reducing expansion (Lin and Kao, 2001). It has become apparent that an excess of free proline has negative or side effects on cell growth or protein functions. Intracellular proline accumulation significantly represses several genes involved in the synthesis of other amino acids or normal morphogenesis in *Arabidopsis* plants (Nanjo et al., 2003). It is also suggested that the proportion of proline to the total amino acids, rather than the concentration, influences the growth of petunia plants (Yamada et al., 2005). Furthermore, the toxicity of proline and its metabolite P5C have been considered to function as signals in stress-induced cell death (Hellman et al., 2000; Maxwell and Davis, 2000;

Morita et al., 2002; Deuschle et al., 2004; Nomura and Takagi, 2004). In *S. cerevisiae*, Maggio et al. (2002) found that intracellular proline accumulation is associated with reduced growth rate. It has also been reported that excess proline might be toxic to yeast cells only when it accumulates in the cytosol (Morita et al., 2002) or might delay yeast cell growth in the presence of ethanol (Takagi et al., 2007). Even at the enzyme level, proline overaccumulation to a concentration as low as 100 mM suppresses the activity of the major chloroplastic enzyme ribulose 1, 5-bis-phosphate carboxylase in higher plants (Sivakumar et al., 1998). These results suggest that intracellular proline must be present at an appropriate level in order to confer stress tolerance. Foliar application of proline was an effective way to improve the salt tolerance of cucumber (Huang et al., 2009). The enhanced salt tolerance could be partially attributed to the improved water status and peroxidase enzyme activity in the leaves.

The method of using exogenous proline proved that mechanism of feedback inhibition of proline synthesis exists in fully turgid plant tissues but not in stressed tissues (Boggess et al., 1976). At this point, enhanced proline oxidation also cannot be ignored. Moreover, this technique emphasized the role of proline as a compatible solute involved in the process of the osmotic adjustment of living organisms.

## 9.6 PROLINE CONTENT AS AN INDICATOR FOR BREEDING PROGRAMS

The existence in plants of quantitative variations in the physiological trait of proline accumulation in response to water or salt stresses has suggested its possible consideration as a selection criterion for breeding programs. This was indeed recommended for cereals growing in Mediterranean environments (Richards et al., 1987). Research performed with 12 paddy genotypes showed a stimulation of proline accumulation in the leaves of plants exposed to salinity (Pandey and Srivastava, 1990). The salinity index of yield showed a significant positive association with proline accumulation, prompting the suggestion of this physiological trait as one of the promising indices for breeding salt-tolerant genotypes in rice. Nitrate reductase activity and proline content were much higher in drought-resistant cultivars of paddy rice; therefore, it was suggested that these two factors may be adopted as an indicator in the identification of drought resistance in rice breeding (Liu et al., 1993). The magnitude of proline response also was suggested for screening alfalfa plants for salt tolerance (Refoufi and Larher, 1989). On the other hand, Ashraf (1989) concluded that proline accumulation cannot be used as an indicator for salt tolerance of black gram and is thus unsuitable for breeding programs. The same is true for soybeans (Moftah and Michel, 1987) and pearl millet (Chandra and Chauhan, 1983) although Petcu et al. (1995) stated that free proline content of the primary leaves appeared to be a good selection criterion in breeding for drought tolerance. Final concentrations of proline accumulated in tobacco plants are advocated as criteria to be used in selecting for drought-tolerant tobacco genotypes as early as the F<sub>1</sub>- or F<sub>2</sub>-generations (1993). Hou et al. (1990) suggested that increase of proline content in plants under soil water stress can be taken as an indicator of drought resistance. Free proline and glycine- $\beta$ -betaine accumulation in the leaves can be used as the possible indicator for drought tolerance in maize genotypes (Moussa and Abdel-Aziz, 2008). Drought resistant sugarcane plants have an enhanced ability to accumulate proline when compared with susceptible lines, making proline content a useful selection criterion (Widyasari and Sugiyarta, 1997). Proline content in storage roots of sugar beet is potentially useful as indicator of situations that lead to decreased yield and quality of the root, such as drought stress (Monreal et al., 2007). Proline accumulation can be used as an indicator of the salt stress imposed on citrus cell lines in suspension cultures (Ferreira and Lima-Costa, 2006). High relative water content (RWC) and Pro over-accumulation were recognized as beneficial drought tolerance indicators and may be used as selection criteria in wheat breeding programs (Bayoumi et al., 2008). Correlation analysis discarded proline as an indicator of drought tolerance (Farshadfar et al., 2008). It can be concluded that, in general, proline accumulation is specific to a genotype, and generalization over different varieties of a crop is not always possible.

## 9.7 CONCLUSIONS

The accumulation of proline in plants subjected to water or salt stress has been observed widely, although not universally. Several possible physiological functions have been ascribed to induced proline accumulation by water shortage. These functions include osmoregulation, a soluble N sink, a signal of senescence, and an indicator of plant resistance to stress. Proline accumulation is a common physiological response to various stresses but is also part of the developmental program in generative tissues. Proline may affect the solubility of various proteins, thus protecting them against denaturation under water-stressed conditions. An increase in the proline content may be associated with either enhanced biosynthesis, with stimulated proline oxidation, or an impaired protein synthesis. In general, proline concentrations are directly proportional to the salinity level or to the intensity of water stress. Genotype variations are very common; however, a positive correlation cannot always be found between proline content and a plant's relative tolerance or susceptibility. Restoring plants to optimal growth conditions, results in a rapid decline in the proline content. Transgenic approaches have confirmed the beneficial effect of proline overproduction during stress, although no consensus was achieved on the exact roles of proline accumulation. Classical gain or loss of function strategies could not bring clear answers, probably because proline also displays the essential role of being a protein component. Environmental stresses such as drought or salinity have multiple targets and proline is believed to play different roles. The balance between biosynthesis and degradation of proline is probably essential in the determination of the osmoprotective and developmental functions of proline. Additional studies are required to elucidate conclusively the role of proline in plant adaptation to stress.

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# 10 Role of Dehydrins in Plant Stress Response

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## 10.1 INTRODUCTION: DEFINITION OF DEHYDRINS, THEIR STRUCTURE, AND FUNCTIONS

### 10.1.1 DEFINITION OF DEHYDRINS

Dehydrins are highly hydrophilic, well-soluble proteins, which belong to a large family of late embryogenesis-abundant (LEA) proteins whose name comes from Galau et al. (1986) who studied them for the first time in cotton (*Gossypium hirsutum* L.) embryos (Dure et al., 1981; Galau and Dure, 1981). They are classified as group 2 LEA (or LEA II) proteins (Bray, 1993; Ingram and Bartels, 1996) or LEA-D11 proteins according to one dehydrin member in cotton embryo (Dure et al., 1989). In Pfam database of protein domains (<http://www.sanger.ac.uk/Software/Pfam>; Bateman et al., 2004), they are simply named dehydrins and have a Pfam number PF00257. They have a relatively high glycine content (greater than 6%) and a hydrophilicity index (Kyte and Doolittle, 1982) greater than 1; thus they can be classified as hydrophilins (Garay-Arroyo et al., 2000; Battaglia et al., 2008). The first reported dehydrin proteins were RAB21, a protein induced by salt and osmotic stress in rice (*Oryza sativa* L.) (Mundy and Chua, 1988) and D-11, a protein accumulating in maturing embryo of cotton (*Gossypium hirsutum* L.) (Baker et al., 1988). At the end of the 1980s, dehydrins were defined as “dehydration-induced proteins” according to their mode of expression (Close et al., 1989). Later, as their sequence characteristics became available, dehydrins were redefined on the basis of their sequential motifs. They were newly defined as proteins possessing at least one copy of a conserved lysine-rich amino acid (aa) sequence—a K-segment—in their molecules (Close, 1996, 1997). Due to this definition based on the presence of a unique amino acid motif, dehydrins can be easily detected by a specific primary antibody raised against the K-segment (Close et al., 1993).

### 10.1.2 CLASSIFICATION OF DEHYDRINS

The K-segment is a lysine-rich aa sequence present in 1–11 copies near to the C-terminus of dehydrin molecules. Consensus amino acid sequence of K-segment in angiosperms is EKKGIME/DKIKEKLPG. Apart from the K-segment, dehydrins can possess other conserved sequential motifs: the tyrosine-rich Y-segment (consensus (V/T)D(E/Q)YGNP) near to the N-terminus and the serine-rich S-segment. S-segment is formed by a stretch of 4–10 serine residues, which are a part of a conserved sequence LHRGS<sub>4–10</sub>(E/D)<sub>3</sub>. This sequence motif can be distinguished by casein kinase II (CK<sub>2</sub>)-type kinases and plays an important role in phosphorylation of serine residues in the S-segment (Svensson et al., 2002; Battaglia et al., 2008). According to the presence of the K-, S-, and Y-segments, dehydrins can be divided into five structural subgroups: K<sub>n</sub>, SK<sub>n</sub>, K<sub>n</sub>S, Y<sub>n</sub>K<sub>n</sub>, and Y<sub>n</sub>SK<sub>n</sub> (Close, 1996, 1997; Campbell and Close, 1997). In addition, dehydrin molecules can contain less conserved sequential motifs rich in glycine and hydrophilic aa called  $\Phi$ -segments.

An alternative classification of dehydrins (group 2 LEA proteins) has recently been proposed by Wise (2003) who divided them into three subgroups—2a, 2b, and 2c—according to protein hydrophilicity, predicted secondary structure, and content of aromatic and charged aa residues. It is interesting that this classification corresponds to different protein stress induction patterns: subgroups 2b and 2c (especially 2c) include predominantly cold-inducible dehydrins while subgroup 2a includes dehydrins not inducible by cold stress.

### 10.1.3 STRUCTURE OF DEHYDRINS

#### 10.1.3.1 Primary Structure

In general, dehydrin aa sequences contain relatively large proportions of glycine (G) and hydrophilic aa, especially threonine (T). In contrast, they nearly lack cysteine (C) and tryptophan (W). These unique sequential characteristics determine high hydrophilicity of dehydrin molecules. Dehydrins are well soluble in various kinds of aqueous buffers and remain soluble in these buffers

even after boiling. This fact can be used for their enrichment in plant tissue extracts (Campbell and Close, 1997; Close, 1997). Dehydrins also exhibit enhanced affinity to detergents such as sodium dodecyl sulfate (SDS). They bind unusually high proportions of SDS when compared with most protein molecules and therefore they appear larger on SDS-PAGE gels (Their electrophoretic mobility is shifted to values typical for larger proteins on SDS-PAGE gels, i.e., their molecular weights determined on the basis of electrophoretic mobility correspond to the proteins with ca 24% higher molecular weight  $M_r$  when compared with the values calculated from dehydrin aa sequence) (Close, 1997; Ismail et al., 1999a).

### 10.1.3.2 Secondary and Tertiary Structure

In aqueous solutions, dehydrin molecules are present in the conformation of random coil, i.e., their molecules form a maximum of hydrogen bonds with neighboring water molecules (intermolecular hydrogen bonds) and a minimum of hydrogen bonds between different aa residues within protein molecules (intramolecular hydrogen bonds). Due to low proportion of intramolecular hydrogen bonds, dehydrin molecules are intrinsically unstructured. Dehydrins share many features with other types of intrinsically disordered/unstructured proteins (IDPs/IUPs) (Tompá, 2002; Tompá et al., 2005): They contain high proportions of hydrophilic aa and change their conformation according to the changes in their ambient microenvironment. Based on several experimental studies (Lisse et al., 1996; Danyluk et al., 1998; Ismail et al., 1999a; Hara et al., 2001; Kovacs et al., 2008; Mouillon et al., 2008), it was confirmed that the decrease in dehydrin hydration status (loss of water molecules in their ambient microenvironment) or addition of high amounts of compatible solutes such as glycerol, detergents such as SDS or salts such as NaCl into dehydrin aqueous solution leads to conformational changes which can be monitored by the technique of far-UV circular dichroism (CD). It was found out that under the conditions of reduced hydration, the regions of the K-segments form left-handed class A2 amphipathic  $\alpha$ -helices, i.e., they form intramolecular hydrogen bonds between different aa residues within the same protein molecules instead of intermolecular hydrogen bonds between aa residues and surrounding water molecules which predominate in random coil conformation. Random coil conformation is relatively symmetrical; thus it does not deviate the plane of linearly polarized light beams significantly, whereas  $\alpha$ -helices are highly asymmetrical, and thus deviate the plane of linearly polarized light. The amphipathicity of the resulting  $\alpha$ -helices is determined by the aa sequence of the K-segment. When  $\alpha$ -helix is formed, negatively charged aa (aa with acidic pI, e.g., D and E) lie on one side of the helix, hydrophobic aa (nonpolar aa, e.g., I and L) lie on the opposite side of the helix, and positively charged aa (aa with basic pI, e.g., K and R) lie on the polar–nonpolar interface (Svensson et al., 2002; Saavedra et al., 2006).

Some researchers (e.g., Soulages et al., 2003) suggest that some dehydrins (e.g., recombinant GmDHN1 from soybean *Glycine max*) do not form classical  $\alpha$ -helical structures after addition of detergents; instead, they rather reveal a left-handed extended poly (L-proline)-type II (PII) helical conformation. Soulages et al. (2003) emphasized that extended PII helical structures contain relatively higher portions of aa residues capable of forming intermolecular hydrogen bonds in comparison with “classical  $\alpha$ -helices” as protein secondary conformation motifs, and thus the extended PII conformations enable dehydrin interaction with membrane surfaces. It should be noted that extended PII helical conformations are typically formed by those protein molecules which have a high content of G (contain G repeats—every third aa is G) and P such as animal collagen.

### 10.1.3.3 Relationships between Dehydrin Structure and Functions

The changes in protein conformation result also in changes in protein function. This phenomenon, which is characteristic for IDPs/IUPs, is called “moonlighting” (Tompá, 2002; Tompá et al., 2005). In the case of IDPs/IUPs, the changes in protein ambient microenvironment, such as availability of water molecules, result in protein conformational and functional changes. The flexibility of protein conformation results in versatility of their functions, which mirror the changes in the protein ambient microenvironment.

In a well-hydrated state, individual macromolecules like proteins or phospholipids in biomembranes are surrounded by a thin layer of highly ordered water molecules bound to the protein or membrane surface via intermolecular hydrogen bonds (“water envelope”). Therefore, the protein or phospholipid macromolecules do not interact with each other directly. During dehydration, the “water envelope” disrupts and the macromolecules then can come into mutual interaction (Israelachvili and Wennerström, 1996). The amphipathic  $\alpha$ -helices can interact with partly dehydrated surfaces of various other proteins and also with surfaces of biomembranes. It has been proposed by Ingram and Bartels (1996) that several K-segments in one dehydrin molecule can form bundles when present in  $\alpha$ -helical conformation thus enhancing their amphipathic character in protein–protein or protein–biomembrane interactions. The binding of dehydrin molecules to the partly dehydrated surface of other protein molecule enhances formation of amphipathic  $\alpha$ -helices in a dehydrin molecule and protects the other protein molecule from further loss of water envelope, which can lead to irreversible changes in the protein conformation, i.e., protein denaturation. It has been suggested that these interactions between partly dehydrated surfaces of dehydrin molecules and partly dehydrated surfaces of other protein molecules and/or biomembranes (observed e.g., by Koag et al., 2003 in case of maize DHN1), which are enabled by the amphipathic nature of the  $\alpha$ -helices, present the basis of dehydrin protective functions such as chaperone function, “molecular shield” function and cryoprotective function. However, direct experimental evidence is still lacking.

Kovacs et al. (2008) investigated chaperone activities of two dehydrin proteins isolated from *Arabidopsis thaliana*, ERD10 and ERD14, and described their protective effects against thermal aggregation of citrate synthase, firefly luciferase, inactivation of lysozyme, and thermal inactivation of alcohol dehydrogenase. However, it turns out that the interactions between dehydrin molecules and other protein molecules are rather nonspecific protein–protein interactions when compared with the interactions of classical chaperones (e.g., small heat-shock proteins). Therefore, some authors describe these dehydrin protective functions based on nonspecific protein–protein interactions as “molecular shield” (Tunnacliffe and Wise, 2007). Under environmental conditions, when cells lose water, the relative spational (three-dimensional) proportions among individual intracellular complexes do also change. These spational changes are also harmful since they can lead to undesirable interactions and aggregation and denaturation of several protein and membranaceous complexes. It has been proposed by several scientists (Tunnacliffe and Wise, 2007; Battaglia et al., 2008) that dehydrins can accumulate to relatively large amounts in various compartments inside the cells under the conditions associated with cellular dehydration. Thus, they can simply act as “space-fillers,” i.e., they can participate in keeping the original, non-harmful distances among individual subcellular complexes. (During dehydration, cell volume diminishes and this process can lead to several undesirable protein interactions resulting in protein denaturation.) Due to their largely unfolded state, capability to bind water, and high level of accumulation, dehydrins can mimic the adverse effects of cellular dehydration and help in keeping the original cell volume, thus preventing structural collapse in the cell. This phenomenon, i.e., accumulation of various kinds of intrinsically unstructured hydrophilic proteins together with low-molecular compatible solutes, is sometimes termed “macromolecular crowding” (Ellis, 2001a,b; Mouillon et al., 2008).

Cryoprotective function, i.e., the ability to protect enzymes from the loss of their activity under freeze-thaw conditions, was first reported by Lin and Thomashow (1992) for COR15a protein, a LEA-3 protein from *A. thaliana* using a lactate dehydrogenase (LDH) assay (EC 1.1.1.27.) developed by Carpenter and Crowe (1988). Since then, a cryoprotective activity has been reported for several dehydrin proteins, e.g., COR85 from spinach (Kazuoka and Oeda, 1994), WCS120 from common wheat (Houde et al., 1995), PCA60 from peach (Wisniewski et al., 1999), CuCOR19 from *Citrus unshiu* (Hara et al., 2001), DHN5 from barley (Bravo et al., 2003), and others. As shown by Reyes et al. (2008), the presence of K-segments is essential for dehydrin cryoprotective activity.

Some dehydrins, which contain relatively large amounts of H, R, and other reactive aa residues on the surface of their folded molecules such as CuCOR15 and CuCOR19 from Satsuma mandarin (*Citrus unshiu*), exhibit also reactive oxygen species (ROS) scavenging and metal ion

binding properties. Both functions are mediated by direct interactions between the aa residue and the ROS species (superoxide anion radical  $O_2^{\cdot-}$ ; singlet oxygen  $^1O_2$ ; hydroxyl radical  $HO^{\cdot}$ ; hydrogen peroxide  $H_2O_2$ ) or the metal ion ( $Co^{2+}$ ;  $Cu^{2+}$ ;  $Fe^{2+}$ ;  $Fe^{3+}$ ;  $Ni^{2+}$ ;  $Zn^{2+}$ ). The interactions of the aa residue with ROS lead to oxidation of the residue, the interactions with metal ions lead to the formation of covalent bonds. Binding of free metal ions prevents the intracellular compounds from excessive ROS formation since free metal ions act as catalyzers of synthesis of various ROS. Dehydrins can thus function also as antioxidants (e.g., CuCOR15 and CuCOR19 in Satsuma mandarin (*Citrus unshiu*)—Hara et al., 2001, 2005), ion sequestrants (e.g., VCaB45 in celery (*Apium graveolens*) vacuoles which binds  $Ca^{2+}$ —Heyen et al., 2002), or metal ion transporters in plant phloem sap (e.g., ITP protein from castor bean (*Ricinus communis*) which binds  $Fe^{2+}$  and  $Fe^{3+}$ —Krüger et al., 2002).

#### 10.1.4 DEHYDRINS AND DEHYDRIN-LIKE PROTEINS WITHIN THE PLANT KINGDOM AND IN OTHER ORGANISMS

From the end of the 1980s until now, hundreds of dehydrin proteins have been described not only in dicotyledonous and monocotyledonous angiosperms such as cotton and rice, but also in other plant taxons including gymnosperms, ferns, lycopods, and mosses. Proteins induced by osmotic stress that contain conserved sequential motifs resembling the dehydrin K-segment have also been found in various groups of cyanobacteria (Close and Lammers, 1993), algae (Li et al., 1998), in mycelia of white truffle *Tuber borchii* from *Ascomycota* (Abba et al., 2006) and in invertebrates, which can survive cellular desiccation in “anhydrobiosis state,” e.g., some nematodes (*Aphelenchus avenae*, *Caenorhabditis elegans*) and bdelloid rotifers (Browne et al., 2002; Tunnaccliffe and Wise, 2007). To the proteins, which resemble dehydrins distantly due to the presence of sequential motifs that can form amphipathic  $\alpha$ -helices analogous to the dehydrin K-segment, belong also  $\alpha$ -synucleins, proteins found in animal neurons where they bind to vesicles with neurotransmitters (Ismail et al., 1999a; Souza et al., 2000).

Dehydrin-like proteins in cyanobacteria and algae usually accumulate inside the cells under conditions associated with osmotic stress. It was proposed that dehydrins protect the cells against excessive water loss which is important namely for those algae that inhabit intertidal zones where they have to face periodical desiccation. Li et al. (1998) detected several dehydrin-like proteins in five species of fucoid algae (*Phaeophyceae*) living in the intertidal zone; unlike dehydrins in higher plants, these proteins were denaturated by boiling.

In seedless plants, i.e., mosses, lycopods, and ferns, dehydrins have been characterized in several species. In contrast to gymnosperms and angiosperms, only one copy of dehydrin gene per genome was found in all these plant taxons (groups). Dehydrins are thus present as single-copy genes in genomes of seedless plants. In mosses, dehydrin genes were sequenced in a desiccation-tolerant moss *Tortula ruralis* (Velten and Oliver, 2001) and in *Physcomitrella patens* (Saavedra et al., 2006). Velten and Oliver found out that during desiccation of *Tortula ruralis*, the level of dehydrin mRNA increases. The researchers have hypothesized that during rehydration, the dehydrin protein Tr288 becomes translated and helps to restore intracellular complexes. Tr288 is therefore also called “rehydrin.” Near to the C-terminus, a conserved sequence which can form an amphipathic  $\alpha$ -helical structure similar to the  $\alpha$ -helices formed by angiosperm dehydrins, occurs. The dehydrin protein characterized in *P. patens*, PpDHNA, has probably a protective function in osmotic stresses since a *P. patens* knockout mutant *dhnA* reveals impaired ability to tolerate these factors (impaired growth on medium containing NaCl or mannitol). In contrast to Tr288, PpDHNA is also up-regulated by ABA. (In *Tortula ruralis*, endogenous ABA was not detected (Oliver et al., 1998).) Dehydrins were also detected in a poikilohydric fern *Polypodium virginianum* (Reynolds and Bewley, 1993) and in a resurrection lycopod *Selaginella lepidophylla* (Iturriaga et al., 2006).

In all gymnosperm and angiosperm species studied so far, more than one copy of dehydrin gene per genome was found. In other words, dehydrins are present as multicopy genes in genomes

of both gymnosperm and angiosperm plants. The different dehydrin genes identified in one plant species usually belong to different structural subgroups and reveal different modes of expression. Dehydrins in gymnosperms differ from their counterparts in angiosperms in the aa sequence of the K-segment. The consensus aa sequence of the K-segment for gymnosperms is (Q/E)K(P/A)G(M/L)LDKIK(A/Q)(K/M)(I/L)PG while for angiosperms is EKKGIMDKIKEKLPG. The first report about detection of dehydrins in gymnosperms comes from Close et al. (1993) who found a positive reaction on a polyclonal primary antibody raised against the angiosperm consensus K-segment in *Ginkgo biloba* and *Pinus taeda*. Later, Jarvis et al. (1996) detected dehydrins in Douglas fir *Pseudotsuga menziesii*. Until now, dehydrins have also been identified in white spruce *Picea glauca* (Richard et al., 2000), in Scots pine *Pinus sylvestris* (Kontunen-Soppela et al., 2000; Wachowiak et al., 2009) in Norway spruce *Picea abies* (Yakovlev et al., 2008), and in cypress *Cupressus sempervirens* (Pedron et al., 2009).

Until now, hundreds of dehydrin genes have been sequenced in different angiosperms including both dicotyledonous and monocotyledonous species. Among dicotyledons, dehydrins have already been characterized in small herbaceous plants such as the thale cress (or mouse-ear cress) *Arabidopsis thaliana* as well as in large, long-living woody species such as silver birch *Betula pendula* (Puhakainen et al., 2004a), downy birch *Betula pubescens* (Welling et al., 2004), beech *Fagus sylvatica* (Jimenez et al., 2008), poplar *Populus tremula* (Renaut et al., 2005), blueberry *Vaccinium corymbosum* (Levi et al., 1999), Satsuma mandarin *Citrus unshiu* (Hara et al., 1999), peach *Prunus persica* (Arora and Wisniewski, 1994), apple tree *Malus domestica* (Wisniewski et al., 2008), *Rhododendron catawbiense* (Wei et al., 2005; Peng et al., 2008), red osier dogwood *Cornus sericea* (Karlson et al., 2003a,b), pistachio *Pistacia vera* (Yakubov et al., 2005), and others.

## 10.2 DEHYDRINS AND PLANT STRESS RESPONSE

### 10.2.1 EXPRESSION OF DEHYDRINS UNDER ENVIRONMENTAL STRESS FACTORS

Dehydrin proteins can be found in small amounts in young plant organs grown under optimum growth conditions and exhibit rapid cell division or cell elongation, e.g., root tips, elongating stems, petioles, etc. (Nylander et al., 2001; Rorat et al., 2004). Analogous to other LEA proteins, dehydrins also accumulate in plant embryos in later stages of their development (embryo maturation and desiccation). Apart from these natural physiological processes, dehydrins become expressed when plants are exposed to various stress factors, which are accompanied by cellular dehydration. Thus, dehydrins can be induced by drought, osmotic stress, enhanced salinity, stresses associated with abscisic acid (ABA) signaling, low-temperature stresses (cold and frost), and also biotic stresses associated with water loss and jasmonic acid signaling such as wounding. All these stresses have a dehydration component. In addition, metal ion binding properties have been reported for some dehydrins (CuCOR15—Hara et al., 2005), which can thus function as chelators and seem to confer plant tolerance to heavy-metal stress (Xu et al., 2008). Dehydrins encoded by one plant genome are usually expressed in different plant tissues (organs) and in response to different developmental and environmental cues; see expression studies in *A. thaliana* by Nylander et al. (2001), Bies-Ethève et al. (2008), Hundertmark and Hincha (2008)—51 *Lea* genes, 10 of them dehydrins; in rice by Wang et al. (2007)—34 *Lea* genes, 8 of them dehydrins identified in Japonica rice Nipponbare; in barley (*Hordeum vulgare*) by Choi et al. (1999) or a more recent work by Tommasini et al. (2008)—13 dehydrin genes.

Under stress conditions, dehydrins accumulate in various compartments inside the cells—nucleus (PCA60 from peach—Wisniewski et al., 1999; WCS120 from common wheat—Houde et al., 1995; phosphorylated forms of maize RAB17 and DHN1—Godoy et al., 1994; Koag et al., 2003; phosphorylated TAS14 in salt-stressed tomato—Godoy et al., 1994; 24 kDa dehydrin in red osier dogwood (*Cornus sericea*)—Karlson et al., 2003a; AmDHN1a from gray mangrove (*Avicennia marina*)—Mehta et al., 2009), nucleolus and euchromatin (TAS14 in salt-stressed tomato (*Lycopersicon*

*esculentum*)—Godoy et al., 1994). Recently, Hara et al. (2009) have reported for CuCOR15, a histidine-rich dehydrin protein, Zn<sup>2+</sup>-dependent DNA and RNA binding affinity. Unlike Cys<sub>2</sub>His<sub>2</sub> zinc-finger transcription factors, the binding affinity of CuCOR15 is not sequence-specific. Thus, Hara et al. (2009) have proposed a general protective function for CuCOR15. Furthermore, dehydrins have been localized to semiautonomous organelles such as outer membrane of mitochondria (Borovskii et al., 2000, 2002; Hara et al., 2003; Carjuzaa et al., 2008), chloroplasts (PCA60 from peach (*Prunus persica*)—Wisniewski et al., 1999; *HaDhn1* transcripts in sunflower (*Helianthus annuus*)—Natali et al., 2007) as well as immature proplastids (Carjuzaa et al., 2008), storage protein bodies and starch-rich amyloplasts (24 kDa dehydrin from downy birch (*Betula pubescens*)—Rinne et al., 1999). Dehydrins have also been localized to various membranaceous structures such as endoplasmic reticulum (CAP85 from spinach—Neven et al., 1993), rough endoplasmic reticulum cisternae (Carjuzaa et al., 2008), vacuole (VCaB45 from celery—Heyen et al., 2002), membranes of lipid vesicles containing acidic phospholipids (maize DHN1—Koag et al., 2003), membranes of protein and lipid bodies (Egerton-Warburton et al., 1997), inner side of plasmalemma (WCOR410 from common wheat—Danyluk et al., 1998) or plasmodesmata of vascular cambium cells (24 kDa dehydrin in *Cornus sericea*—Karlson et al., 2003a). Abu-Abied et al. (2006) have detected *A. thaliana* ERD10 in association with actin stress fibers in transformed rat fibroblasts. They have also reported a protective function of GFP-ERD10 on actin cytoskeleton in latrunculin-treated leaves of *Nicotiana benthamiana*. Taken together, it can be stated that dehydrins are intracellular proteins induced by stresses resulting in cellular dehydration. Until now, dehydrins have not been detected in cell walls.

At tissue and organ level, dehydrins accumulate mainly in those tissues and organs which are exposed to rapid changes in water content, i.e., outer leaves of inflorescence buds, epidermis in herbs or bark in woody plants, stomatal guard cells (e.g., Rab18 from *A. thaliana*—Nylander et al., 2001; *HaDhn1* transcripts from sunflower *Helianthus annuus*—Natali et al., 2007), adventitious root primordia, root apices (e.g., Lti29/ERD10 and ERD14 in *A. thaliana*—Nylander et al., 2001), and vascular tissues. In vascular tissues, dehydrins usually accumulate in living cells in the vicinity of vascular elements, especially in xylem ray parenchyma cells and phloem ray parenchyma cells (Godoy et al., 1994; Bravo et al., 1999; Wisniewski et al., 1999, 2008; Yakubov et al., 2005; Natali et al., 2007; Yakovlev et al., 2008).

For some dehydrin genes, an alternative splicing was reported, e.g., *VvDHN-1a* and *VvDHN-1b* transcripts of *VvDHN1* gene in grapevine (*Vitis vinifera*) (Xiao and Nassuth, 2006), *AmDHN1* and *AmDHN1a* transcripts of a dehydrin gene in gray mangrove (*Avicennia marina*) (Mehta et al., 2009), or *OsLea7* and *OsLea8* genes in Japonica rice (*Oryza sativa* cv. *japonica*) (Wang et al., 2007). For some dehydrin proteins, two types of posttranslational modifications—phosphorylation and O-glycosylation—under stress conditions have been reported. While phosphorylation has been quite widely reported, especially for dehydrins with the S-segment, and several functions for phosphorylated dehydrin proteins have been proposed, O-glycosylation has been reported only for some dehydrins in blueberry and pistachio (Levi et al., 1999; Yakubov et al., 2005) and no specific functions for glycosylated forms of these proteins have been suggested. Phosphorylation of the S-segment by casein kinase II (CK2)-type kinases has been shown to be associated with dehydrin translocation from cytoplasm into nucleus (e.g., RAB17 in maize (*Zea mays*)—Godoy et al., 1994; Jensen et al., 1998; Riera et al., 2004; TAS14 in tomato (*Lycopersicon esculentum*)—Godoy et al., 1994; DHN-5 in durum wheat (*Triticum turgidum* ssp. *durum*)—Brini et al., 2007a; however, nuclear localization has also been reported for some dehydrins that lack the S-segment (e.g., wheat WCS120 or peach PCA60; Houde et al., 1995; Wisniewski et al., 1999). For *A. thaliana* acidic dehydrins COR47, ERD10, and ERD14 and for celery (*Apium graveolens*) vacuole-located dehydrin VCaB45, it has been reported by Alsheikh et al. (2003, 2005) and by Heyen et al. (2002), respectively, that phosphorylation is necessary for Ca<sup>2+</sup> binding properties of these proteins. Recently, *in vitro* phosphorylation has also been reported for K<sub>n</sub>-type dehydrin Lti30 from *A. thaliana* by Eriksson and Harryson (2009). The phosphorylation of K<sub>n</sub>-type dehydrins is provided by a different kinase than phosphorylation of dehydrins with an S-segment.

When dehydrin expression at transcript (mRNA) and protein level is compared, several differences can be found. Zhu et al. (2000) investigated expression of barley *Dhn5* gene at transcript and protein level in plants grown under field conditions and found out that either only transcript or only protein molecules can be present in the sample. Different dynamics of dehydrin transcript and protein accumulation during a time course of a stress treatment has also been described. For example, Ganeshan et al. (2008) compared wheat dehydrin (*Wcs120*, *Wcor410*) expression at transcript and protein levels during a long-term cold acclimation (CA) and found out that the peak in transcript accumulation precedes the peak in protein accumulation during the time course of CA treatment (2 days of CA versus 56 days of CA at transcript and protein levels, respectively).

Recently, dehydrin expression in various plant species and their functions under different stress conditions have been reviewed by Svensson et al. (2002), Allagulova et al. (2003), Rorat (2006), and specifically for cold stress by Kosová et al. (2007). In NCBI database (<http://www.ncbi.nlm.nih.gov/>), 1090 dehydrin protein sequences from 133 plant species were available in July, 2009. In the following paragraphs, expression of dehydrins and their specific functions in different plants under different stress conditions will be characterized according to the following scheme: First, a brief characteristics of each stress factor will be given. Second, signaling pathways which cooperate in induction of dehydrin expression under a given stress factor will be characterized. Third, examples of dehydrins expressed in various plant species under a given stress factor will be listed and their specific functions (if known) will be discussed.

## 10.2.2 DROUGHT STRESS, EVAPORATION

### 10.2.2.1 Physiological Aspects of Drought Stress

Drought is one of the major abiotic stress factors, which significantly limit plant growth and agronomic production in many areas worldwide. Drought, i.e., a shortage of rainfall, leads to the decrease in soil water potential (gravimetric soil water content), which is also often accompanied by a decrease in air relative humidity resulting in an enhanced rate of leaf transpiration. The decreased soil water potential draws water from ambient environment with higher water potential including plant cells. Plant acclimation to drought stress which tries to eliminate excessive water loss lies in osmotic adjustment, i.e., a decrease of cell water potential in order to diminish the difference in water potential between the plant cell and the ambient soil. Osmotic adjustment is associated with accumulation of low-molecular well-soluble metabolites collectively called compatible solutes (low-molecular saccharides, monosaccharides: glucose, fructose; disaccharides: sucrose, oligosaccharides raffinose, stachyose, and verbascose; sugar alcohols: mannitol, pinitol, sorbitol; quaternary ammonium compounds called betaines: alanine betaine, glycine betaine; imino acid proline; polyamines spermine, spermidine, putrescine) and with relatively high-molecular hydrophilic proteins inside the cells. Among hydrophilic proteins (hydrophilins), several LEA proteins including dehydrins accumulate to relatively high extents (amounts) in various plant cells during the process of osmotic adjustment. Osmotic adjustment lies in fact in the decrease of one major component of water potential—osmotic potential, which is determined by concentration (activity) of dissolved compounds in a solute (osmotic potential is defined as a negative value of osmotic pressure). Other components of cell water potential, i.e., pressure (or turgor) potential, which is determined by a hydrostatic pressure of cytosol against cell wall, gravitational potential, which is determined by position of water in gravitational field, and matrix potential, which is determined by adhesive forces of water-to-cell surfaces, are not significantly affected by accumulation of compatible solutes.

In soil water potential (relative air humidity) as well as in leaf water potential, diurnal changes have been observed, with maximum values before dawn and minimum values at midday (noon). It is becoming evident that these changes are mirrored in the expression of several drought-inducible hydrophilic proteins including dehydrins (e.g., Cellier et al., 2000).



### 10.2.2.2 Signaling Pathways Involved in Drought-Inducible Dehydrin Gene Expression

Drought-inducible dehydrins contain ABA-responsive elements (ABRE), C-repeat/drought-responsive/low-temperature-responsive elements (CRT/DRE/LTRE), myeloblastosis (MYB) and myelocytomatosis (MYC) regulatory elements in their promoter regions. Their expression is thus regulated by both ABA-dependent and ABA-independent signaling pathways. ABA-dependent signaling pathways include either bZIP transcription activators named ABFs or AREBs (ABRE-binding factors), which bind to ABRE elements, homologues of *A. thaliana* CBF4/DREB1D transcription activator, which bind to CRT/DRE/LTRE elements, and MYBFs and MYCFs, which bind to MYB and MYC promoter elements. ABA-independent signaling pathways include homologues of *A. thaliana* DREB2A and DREB2B transcription activators, which bind to CRT/DRE/LTRE elements (for review on stress signaling pathways and regulatory elements in the promoters of *Corl/Lea* genes, see, e.g., Bray, 1997; Zhu, 2002; Chinnusamy et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 1997, 2000; Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006).

### 10.2.2.3 Dehydrin Expression under Drought

#### 10.2.2.3.1 Dehydrin Expression in Mature Seeds under Drought

Dehydrins as LEA proteins accumulate in plant seeds which undergo desiccation during a physiological process of maturation drying. For example, Jiménez et al. (2008) and Kalembe et al. (2009) reported accumulation of dehydrins during desiccation of beech (*Fagus sylvatica*) embryos and seeds. It is well known that mature plant embryos in seeds that undergo desiccation during their maturation are tolerant to drought. The seeds that undergo maturation drying are called orthodox seeds (e.g., barley, beech, maize, or rice). In contrast, seeds of some species, e.g., tropical wetland plants such as gray mangrove (*Avicennia marina*), but also some temperate woody plants such as horse chestnut (*Aesculus hippocastanum*) or oak (*Quercus* sp.), do not undergo maturation drying. They remain metabolically active throughout embryo maturation and can germinate already before shedding from the parent plant. These seeds are called recalcitrant seeds. (To get more information about seed recalcitrance, see e.g., Berjak and Pammenter, 2008.) Some researchers have shown that these two physiologically different types of seeds—orthodox and recalcitrant—differ also in accumulation of LEA proteins including dehydrins. Farrant et al. (1992) have reported an absence of dehydrins in later stages of seed development in gray mangrove (*Avicennia marina*) recalcitrant seeds. Similarly, Han et al. (1997) have shown that the susceptibility to desiccation in recalcitrant seeds of *Trichilia dregeana* may be due to their inability to accumulate sufficient amounts of dehydrins, especially after the beginning of germination. In contrast to these studies, no differences in dehydrin levels were found between desiccation-tolerant orthodox seeds of rice (*Oryza sativa*) and recalcitrant seeds of *Zizania palustris* during germination (Bradford and Chandler, 1992). In young seedlings, an ABA-induced dehydrin expression was observed in tolerant *Cynanchum komarovii* plantlets upon severe dehydration stress (Yang et al., 2007). It is well known that dehydrin accumulation in plant embryos and seedlings is regulated by ABA. However, dehydrin *Mat1* in soybean (*Glycine max*) seedlings is induced by dehydration, but not by ABA—that is quite unusual for dehydrin expressed under drought conditions (Whitsitt et al., 1997).

#### 10.2.2.3.2 Dehydrin Expression in Plants under Drought

Dehydrins play an important role not only in maturing plant embryos, but also in later stages of plant development during plant response to stress factors associated with cellular desiccation. According to tolerance to cellular desiccation, plants can be divided into two major groups: poikilohydric plants and homoiohydric plants. Poikilohydric plants are generally much more tolerant to cellular dehydration than homoiohydric plants due to small vacuoles and relatively low vacuole to cytoplasm ratio when compared with homoiohydric plants (about poikilohydry and homoiohydric in plants, see review by Proctor and Tuba, 2002).

Poikilohydric plants include predominantly various desiccation-tolerant seedless plants such as desiccation-tolerant mosses, lycopods from the genus *Selaginella* and ferns from the genus

*Polypodium*. However, they also include a few desiccation-tolerant angiosperms which have cells with small vacuoles and can recover after up to 95% (practically total) water loss. These plants are sometimes called “resurrection plants” —e.g., *Craterostigma plantagineum*, *Ramonda serbica*, etc. (Scott, 2000).

In desiccation-tolerant moss *Tortula ruralis*, dehydrin Tr288 with several repeats of K-segments in its C-terminal domain was detected by Velten and Oliver (2001). These researchers found out that during dehydration, particles of Tr288 mRNP accumulate in desiccating cell cytoplasm. The researchers have hypothesized that Tr288 protein could become expressed (synthesized) during subsequent cellular rehydration rather than during dehydration. Therefore, Tr288 can be better characterized as a “rehydrin.” Dehydrins have also been described in *Polypodium virginianum* (Reynolds and Bewley, 1993) and in *Selaginella lepidophylla* (Iturriaga et al., 2006). In *Craterostigma plantagineum*, an *in vitro* unfolded dehydrin Dsp16 has been studied (Piatkowski et al., 1990; Lisse et al., 1996).

Dehydrins also accumulate in homoiohydric plants when they are exposed (subjected) to drought stress. Several studies have shown that dehydrin accumulation during plant vegetative growth correlates with their drought tolerance. Labhili et al. (1995) and Brini et al. (2007a) have shown that dehydrin accumulation confers tolerance to drought and salt stress in durum wheat (*Triticum turgidum* ssp. *durum*). The latter authors have also shown nuclear localization of a stress-inducible dehydrin protein DHN-5 and they have also detected differences in phosphorylation pattern of DHN-5 between two Tunisian cultivars of durum wheat. A sensitive cultivar revealed only a low level of dehydrin phosphorylation while a highly tolerant cultivar exhibited high level of dehydrin phosphorylation. DHN-5 from durum wheat is  $Y_nSK_n$  type dehydrin sequentially similar to bread wheat Rab15, barley DHN3, DHN4, and DHN9, or maize Rab17. In wild barley (*Hordeum vulgare* ssp. *spontaneum*), Suprunova et al. (2004) have observed expression of several  $Y_nSK_n$ -type dehydrins (especially *Dhn1* and *Dhn6*, but also *Dhn3* and *Dhn9*) associated with enhanced drought tolerance.

Similarly, Pelah et al. (1997) have observed a positive relationship between dehydrin accumulation and drought tolerance in aspen (*Populus tremula*). Analogously, Tabaei-Aghdaei et al. (2000) compared dehydrin protein accumulation in crown tissues of two wheatgrass species revealing different levels of drought tolerance—a drought-tolerant *Agropyron desertorum* and a less tolerant *Lophopyrum elongatum*—under drought stress (6 days without watering) and the researchers detected much higher levels of dehydrin polypeptides in the drought-tolerant *A. desertorum* than in *L. elongatum*. Volaire (2002) found association of 22–24 kDa dehydrin accumulation with enhanced tolerance to drought and acquisition of summer dormancy in leaf bases of two contrasting varieties of cocksfoot (*Dactylis glomerata*), one of Mediterranean Moroccan origin (a drought-tolerant variety) and the other of oceanic French origin (a drought-sensitive variety). In contrast, no differences were observed at transcript level (Voltaire et al., 1998). These results suggest an existence of different regulatory mechanisms between *Dactylis glomerata* varieties with different drought tolerance at posttranscriptional level.

Cellier et al. (1998) have observed higher transcript levels of dehydrin genes *HaDhn1* and *HaDhn2* in a drought-tolerant cultivar of sunflower (*Helianthus annuus*) when compared with a drought-sensitive one. The accumulation of *HaDhn1* and *HaDhn2* transcripts correlates with plant leaf water potential when the cultivars were compared under the same gravimetric soil water content. Cellier et al. (2000) have also observed diurnal changes in *HaDhn1* gene expression in sunflower plants subjected to drought with a maximum *HaDhn1* expression at midday (noon) which mirrored the changes in leaf water potential. In contrast, *HaDhn2* gene showed no significant diurnal changes in its expression pattern.

### 10.2.3 SALINITY AND OSMOTIC STRESS

#### 10.2.3.1 Brief Characteristics of Salinity Stress

High salinity means enhanced salt ion concentrations in soil water, which is taken up by plants. Plants as well as other organisms actively regulate cytoplasmic (intracellular) ion concentrations, especially concentrations of  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ , and  $Cl^-$  and phosphate ions via ATP-dependent ion

channels (pumps) in plasmalemma, tonoplast, and membranes of endoplasmic reticulum. According to tolerance to enhanced cytoplasmic ion concentrations, plants can generally be divided into glycophytes, which do not tolerate enhanced (increased) cytoplasmic ion concentrations, and halophytes, which tolerate (or even require) enhanced cytoplasmic ion concentrations. Glycophytes include most economically important plant crop species such as barley, wheat, maize, or rice, but also a model plant *Arabidopsis thaliana*. Halophytes include mostly plants living on sea coasts where they are in permanent contact with salt water—e.g., *Salsola kali*, *Salicornia*, or *Arabidopsis* salt-tolerant relative *Thellungiella salsuginea* which is used as a model plant for studies of plant salt-tolerance mechanisms (Amtmann, 2009).

In case of glycophytes, high ion concentrations in soil water result in decreased osmotic potential of soil solution relative to cytoplasmic osmotic potential and this difference triggers water from plant cell cytoplasm. Thus, high salinity has a similar effect on glycophytes as a drought stress. Therefore, compatible solutes such as proline, sugar alcohols, polyamines, low molecular saccharides (products of starch degradation—maltose, glucose) as well as hydrophilic proteins (dehydrins) accumulate in cell cytoplasm upon salt stress.

#### 10.2.3.2 Salt-Inducible Dehydrins

Dehydrin expression under salt stress has predominantly been studied in several economically important glycophytes whose production is strongly reduced by enhanced soil salinity. Only a few studies have been carried out on relatively salt-tolerant plant species such as *Avicennia marina* or *Lophopyrum elongatum* (Tabaei-Aghdai et al., 2000; Mehta et al., 2009).

Godoy et al. (1994) have studied tissue and subcellular localization of dehydrin TAS14 in salt-stressed tomato (*Lycopersicon esculentum*) and found its major accumulation in nucleus and nucleolus in the cells of xylem ray parenchyma neighboring the vascular tissue. The protein accumulated especially in stems and leaves and only a transient accumulation was detected in roots. Godoy et al. (1994) also found out that TAS14 is a phosphoprotein *in vivo* and that it could be phosphorylated by both casein kinase II and cAMP-dependent protein kinase *in vitro*.

Moons et al. (1995) have detected several dehydrins and LEA 3 proteins of various molecular mass (dehydrins of 24 kDa, 35 kDa, and 50 kDa) in roots of salt-tolerant Indica rice (*Oryza sativa* cv. indica) varieties Pokkali and Nona Bokra in response to salt stress (50 mM NaCl) and exogenous ABA treatment (20  $\mu$ M or 100  $\mu$ M ABA; pH 5.6). Similarly, Gulick and Dvořák (1992) have detected expression of one dehydrin in roots of salt-tolerant wheatgrass species *Lophopyrum elongatum* in response to salt stress (250 mM NaCl). Masmoudi et al. (2001) found differences in the expression of *DHN-5* gene (*Tdsi-5* cDNA clone) in roots and leaves of two cultivars of durum wheat (*Triticum turgidum* ssp. *durum*) with marked differences in their salt tolerance when the seedling plants were exposed to salt stress (compost soil supplemented with 200 mM NaCl). Later, Brini et al. (2007a) also reported differences in DHN-5 phosphorylation pattern between the same cultivars exposed to the same type of salt stress with large portions of phosphorylated forms of DHN-5 in a tolerant cultivar and much less amounts of phosphorylated DHN-5 in a salt-sensitive one. In contrast, Tabaei-Aghdai et al. (2000) detected no significant differences in the accumulation of dehydrin polypeptides in crown tissues of two wheatgrass species—a salt-tolerant *Lophopyrum elongatum* and a less tolerant *Agropyron desertorum*—when the plants were subjected to a high salt stress (daily exposure to 75 or 150 mM NaCl for 6 days).

Accumulation of dehydrins in response to salt and osmotic stress has also been reported in woody plants. In poplar *Populus euramericana*, enhanced levels of dehydrin transcript of *PeuDhn1* (an SK<sub>n</sub> type dehydrin) were observed by Caruso et al. (2002) in response to salt stress (1.36 g L<sup>-1</sup> NaCl) or osmotic stress (hydroponic cultivation with 50 g L<sup>-1</sup> PEG 6,000). The level of expression of this gene seemed to correlate with leaf osmotic potential. Mehta et al. (2009) found a rapid up-regulation of *AmDHN1* gene (a YSK<sub>n</sub> type dehydrin) by 500 mM NaCl in gray mangrove (*Avicennia marina*), a pantropical mangrove species inhabiting coastal locations with excess salinity.

## 10.2.4 DEHYDRINS AND LOW-TEMPERATURE STRESS (COLD AND FROST)

### 10.2.4.1 Brief Characteristics of Low-Temperature Stresses (Cold and Frost)

Biennial and perennial plants inhabiting locations in higher latitudes and/or altitudes have to cope with relatively long periods of low temperatures (cold and frost) during winter. Annuals that survive winter as seeds often have to resist low temperatures as seedlings in early spring. In higher latitudes, periods of low temperatures are associated with short-day photoperiods, which are sensed by plants and function as a signal which precedes low-temperature stress (e.g., in initiation of winter dormancy in woody perennials). Plant winter hardiness has several components, e.g., desiccation tolerance, tolerance to the effects of snow cover, tolerance to flooding or repeated freeze-thaw cycles. Frost tolerance, i.e., plant ability to withstand adverse effects of low-temperature stress (especially effects of subzero temperatures, i.e., frost), presents a major component of plant winter hardiness. According to tolerance to low temperatures, plants can generally be divided into three groups: (1) chilling-sensitive plants which are susceptible to low above-zero temperatures (chilling: usually defined as a range between 0°C and +12°C (+15°C)—these plants include many crops of tropical and subtropical origin, such as cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), maize (*Zea mays*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), tropical legume crops such as cowpea (*Vigna unguiculata*) and chickpea (*Cicer arietinum*); (2) chilling-tolerant, but frost-sensitive plants, which are tolerant to chilling, but severely damaged by frost; and (3) frost-tolerant plants that can survive freezing temperatures in vegetative stage—these include especially winter varieties of *Triticeae* cereals, i.e., winter barley (*Hordeum vulgare*), winter wheat (*Triticum aestivum*), or winter rye (*Secale cereale*) (Levitt, 1980; Sakai and Larcher, 1987).

However, in most frost-tolerant plants, frost tolerance is usually not a constitutive trait, but it can be induced by low, but above-zero temperatures (cold). This adaptive process is called cold acclimation (CA). Different aspects of CA are reviewed in Levitt (1980), Sakai and Larcher (1987), Guy (1990), Pearce (1999), Thomashow (1999), Xin and Browse (2000), and others. CA process leads to enhancement of plant winter hardiness and frost tolerance. Frost, i.e., freezing of water—formation and growth of ice crystal nuclei—is accompanied by cellular dehydration. Therefore, plant cold acclimation leads to adaptive responses in order to minimize adverse effects of cellular dehydration. These responses are associated with accumulation of compatible solutes and hydrophilic proteins including dehydrins in various intracellular compartments.

### 10.2.4.2 Signaling Pathways Involved in Dehydrin Expression under Cold and Frost

Similar to drought stress, dehydrins expressed under cold are regulated by both ABA-dependent and ABA-independent regulatory pathways. ABA-dependent pathways include ABRE regulatory elements in dehydrin promoters, which function as binding sites for bZIP ABRE-binding transcription factors (AREBs or ABFs). The main ABA-independent cold-inducible signaling pathway is the CBF/DREB1 pathway. The CBF/DREB1 transcription activators possess AP2 DNA-binding domain flanked by two unique sequence motifs (called signature motifs), which can bind to CRT/DRE/LTRE elements in dehydrin (and other *Cor* gene) promoter region. The up-regulation of several *Cor* gene expression as a consequence of CBF gene overexpression was first reported by Jaglo-Ottosen et al. (1998) in *A. thaliana*. In the genome of *A. thaliana*, three predominantly cold-inducible *CBF/DREB1* genes—*CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A*—were characterized which are tandemly arranged on chromosome four. (The fourth member of *CBF/DREB1* gene family—*CBF4/DREB1D*—is predominantly drought- and ABA-inducible—Haake et al., 2002.) Currently, homologues of *A. thaliana CBF/DREB1* genes have been sequenced and further characterized in several other species, especially in cereals from the tribe *Triticeae* (e.g., Skinner et al., 2005, 2006; Miller et al., 2006; Badawi et al., 2007; Francia et al., 2007) where the majority of *CBF* genes was mapped to *Frost-resistance 2* (*Fr-2*) locus, previously characterized as a major QTL determining frost tolerance level and *Cor* gene expression in *Triticeae* (e.g., Vágújfalvi et al., 2000, 2003). The conformation of the AP2 domain appears to be essential for binding of the CBFs to CRT/DRE/LTRE promoter

elements and activation of *Cor* gene expression. Recently, Knox et al. (2008) have described an allelic variation in *TmCBF12* gene located to *Fr-A<sup>m</sup>2* locus in einkorn wheat (*Triticum monococcum*). A freezing-sensitive line of *T. monococcum* possesses *TmCBF12* allele with a deletion of a few aa residues in the AP2 domain of the protein. As a consequence, *TmCBF12* cannot bind to CRT/DRE/LTRE elements in *Cor* gene promoters.

Recently, Bassett et al. (2009) have reported conformational transitions in the promoter regions of cold-inducible peach dehydrin genes *PpDhn1* and *PpDhn3*. They have hypothesized that these cold-inducible conformational transitions may strongly influence cold-inducible expression of these dehydrins.

#### 10.2.4.3 Low Temperature-Inducible Dehydrins and Their Features

Low temperature-induced dehydrins exhibit various protective functions: cryoprotective function, antifreeze activity, cold-inducible phosphorylation, metal ion binding, and ROS scavenging activities.

A cryoprotective activity has been reported for several cold-inducible dehydrins, e.g., COR85 from spinach (Kazuoka and Oeda, 1994), WCS120 from common wheat (Houde et al., 1995), PCA60 from peach (Wisniewski et al., 1999), CuCOR19 from *Citrus unshiu* (Hara et al., 2001), DHN5 from barley (Bravo et al., 2003). When LDH assay is used, these proteins exhibit significant cryoprotective activity, which is sometimes stronger than a protective activity of several widely used sugar cryoprotectants such as sucrose or cryoprotective proteins such as bovine serum albumin (BSA). A protective effect on the activity of  $\alpha$ -amylase (EC 3.2.1.1) under cold has been reported by Rinne et al. (1999) for a 24 kDa dehydrin from downy birch (*Betula pubescens*).

An antifreeze activity, i.e., modification of ice crystal growth and thermal hysteresis during freezing of protein aqueous solution was first reported for PCA60, a Y<sub>2</sub>K<sub>9</sub> dehydrin isolated from bark tissue of peach (*Prunus persica*) (Arora and Wisniewski, 1994; Wisniewski et al., 1999) and encoded by *PpDhn1* gene. At high concentrations (micromolar to millimolar) of antifreeze proteins, ice crystal growth is inhibited along the *a*-axes while the *c*-axis becomes the preferred direction of growth. As a consequence, instead of flat round crystals formed by ice crystal nuclei in pure water, ice crystals of a shape of hexagonal bipyramids are formed in aqueous solutions containing proteins with an antifreeze activity. Moreover, the proteins with an antifreeze activity also decrease the freezing point of aqueous solutions in which they are dissolved; this phenomenon is called thermal hysteresis. In plants, antifreeze activity was first reported in 1992 for a specific group of proteins named antifreeze proteins (AFPs) (Griffith et al., 1992). PCA60 thus shares this function with AFPs, which also accumulate in the bark of woody perennials during winter dormancy (for review on AFPs, see e.g., Griffith and Yaish, 2004). Unlike dehydrins, AFPs are apoplastic proteins that accumulate in intercellular spaces where they inhibit growth and recrystallization of ice. Recently, a boiling-stable antifreeze protein revealing a sequence homology to dehydrins (containing several lysine-rich sequences and a Y-segment) was isolated from cold-acclimated bark tissue of *Forsythia suspensa* (Simpson et al., 2005).

Cold-induced phosphorylation of the S-segment has been reported for acidic SK<sub>n</sub>-type dehydrins COR47, ERD10 and ERD14 in *A. thaliana* (Alsheikh et al., 2003, 2005). When phosphorylated, these proteins can bind Ca<sup>2+</sup> ions, and Alsheikh et al. have speculated that they may act as calcium buffers or sugar chaperones under these conditions. It is also well known that Ca<sup>2+</sup> ions act as second messengers in cold-inducible signaling pathways.

Radical-scavenging and metal ion binding properties of dehydrin proteins have already been discussed in Section 10.1.3.3.

##### 10.2.4.3.1 Chilling-Induced Dehydrin Expression in Mature Seeds

An increase in dehydrin transcript or protein level has also been observed in seeds exposed to chilling; e.g., *FsDhn1* gene in European beech (*Fagus sylvatica*; Jiménez et al., 2008). These researchers have also found out that the expression of *FsDhn1* gene in beech seeds is strongly up-regulated

by ABA treatment. Ismail et al. (1999b) have reported for an extremely chilling-sensitive tropical legume crop *Vigna unguiculata* that seeds of a relatively chilling-tolerant line 1393-2-11 contained DHN1 (Y<sub>2</sub>K-type), which positively correlated with seedling emergence under mild cold conditions (14°C), while the DHN1 was absent in genetically related, but chilling-sensitive line 1393-2-1. Yao et al. (2005) have shown that expression of dehydrin genes *BnDHN1* and *BjDHN1* (both homologous Y<sub>3</sub>SK<sub>2</sub> dehydrins) in cold-treated seeds of *Brassica napus* and *Brassica juncea*, respectively, enhances plant cold tolerance during seedling emergence. These findings are analogous to the situation in *Arabidopsis thaliana* where RAB18 protein (also Y<sub>2</sub>SK<sub>2</sub> dehydrin) is known to accumulate in mature seeds and to confer seedling tolerance to cold stress (Lång and Palva, 1992; Nylander et al., 2001; Hundertmark and Hinch, 2008).

#### 10.2.4.3.2 Dehydrin Expression in Plants upon Cold

In many plants, accumulation of dehydrin transcripts and/or proteins has been observed during cold acclimation process (CA), which often precedes frost in nature. During CA, dehydrin accumulation was reported for various intracellular compartments, e.g., plasmalemma in case of WCOR410 in common wheat *Triticum aestivum* (Danyluk et al., 1998), endoplasmic reticulum in case of CAP85 in spinach (Neven et al., 1993), nucleus in case of WCS120 in common wheat (Houde et al., 1995) or PCA60 in peach (*Prunus persica*), chloroplasts in case of peach PCA60 (Wisniewski et al., 1999), amyloplasts in case of 24 kDa dehydrin from downy birch *Betula pubescens* (Rinne et al., 1999), mitochondria in case of dehydrins in wheat (Borovskii et al., 2000, 2002). At tissue level, dehydrin accumulation under cold stress was observed predominantly in epidermal cells and in xylem ray parenchyma cells, i.e., in tissues in neighborhood of large extracellular spaces where ice crystals can be formed during freezing. This tissue distribution was observed for cold-inducible dehydrins in both dicotyledons and monocotyledons for *Arabidopsis thaliana* (Nylander et al., 2001) as well as for barley (Bravo et al., 1999). In citrus trees, some dehydrins have been reported to accumulate in fruit flavedo tissue after a rapid temperature change (chilling after a brief heat shock). This pattern of dehydrin induction was reported for CsDHN in orange *Citrus sinensis* and for COR15 and CpDHN in grapefruit *Citrus paradisi* (Porat et al., 2002, 2004).

Cold-inducible dehydrins have been studied in many herbaceous and woody plants. Currently, the most studied plant is *Arabidopsis thaliana* in which a total of 51 *Lea* genes have been identified (Hundertmark and Hinch, 2008), 10 of them represent dehydrins (Nylander et al., 2001). For five dehydrins—*Cor47*, *Erd10/Lti29*, *Erd14*, *Lti30/Xero2*, and *Rab18*, a cold-inducible expression has been reported (Nylander et al., 2001). Three cold-inducible dehydrin genes, *Cor47*, *Erd10/Lti29*, and *Erd14* encode acidic SK<sub>n</sub>-type proteins while *Lti30* encodes a K<sub>n</sub>-type protein and *Rab18* encodes predominantly drought- and ABA-inducible (but also cold-inducible) basic Y<sub>n</sub>SK<sub>n</sub>-type protein. While the first four dehydrin proteins accumulate only in various non-seed tissues (predominantly vascular tissues, but also root tips in case of ERD10, or anthers in case of Lti30), RAB18 accumulates also in mature embryos.

A positive effect of dehydrin transcript and/or protein accumulation upon CA conditions on plant tolerance to cold and/or frost has been reported for many cold-inducible dehydrins in various plant species, e.g., for CpDHN1 in *Cicer pinnatifidum*, which is a cold-tolerant wild relative of chickpea *Cicer arietinum* (Bhattarai and Fettig, 2005), CAS15 in alfalfa *Medicago sativa* (Monroy et al., 1993), CAS15 and CAS18 dehydrins in cell suspension culture of alfalfa *Medicago falcata* (Wolfrain et al., 1993), WCS120 in common wheat *Triticum aestivum* (Houde et al., 1992a,b; Vítámvás et al., 2007), DHN5 in barley (Close et al., 1995; Kosová et al., 2008), etc.

#### 10.2.4.3.3 Dehydrin Expression in Woody Plants during Winter Dormancy

In woody perennials, dehydrins accumulate predominantly during winter dormancy. Winter dormancy is a complex physiological process that is aimed at the enhancement of winter hardiness (overwintering) in woody perennials and that is induced by low temperatures (both cold and frost) and short-day photoperiods during autumn. Processes of cold acclimation and winter dormancy in

woody plants were reviewed recently by Welling and Palva (2006). The process of winter dormancy acquisition is associated with several changes in plant metabolism including accumulation of various hydrophilic proteins (dehydrins).

Wisniewski et al. (2008) studied expression of dehydrin EST homologous to *Arabidopsis Xero2* gene in young apple trees (*Malus × domestica* cv. “Royal Gala”) under cold (24 h at 5°C) and found a high increase in dehydrin expression in bark and xylem tissues and only a mild enhancement in leaves. Therefore, it can be concluded that dehydrin expression in woody plants subjected to cold is tissue-specific. In bark tissues, several hydrophilic proteins (e.g., bark storage proteins, 70 kDa heat-shock proteins, dehydrins) accumulate under cold acclimation (e.g., Wetzel et al., 1989; Wisniewski et al., 1996; Pagter et al., 2008).

For several dehydrins accumulating during winter dormancy, induction by short-day photoperiods has been reported—e.g., *BpLti36* gene in silver birch (*Betula pendula*) (Puhakainen et al., 2004a), *BpuDHN1* (a dominant effect of SD induction) and *BpuDHN2* (only a small effect of SD induction) genes in pubescent (downy) birch (*Betula pubescens*) (Welling et al., 2004), *BbDhn7* gene in blueberry (*Vaccinium corymbosum*) (Dhanaraj et al., 2005), spliced forms of *DHN-1* genes in *Vitis riparia* and *Vitis vinifera* (Xiao and Nassuth, 2006), 24 kDa dehydrin in red osier dogwood (*Cornus sericea*) (Karlson et al., 2003b), or *PaDhn1* and *PaDhn6* genes in Norway spruce (*Picea abies*) (Yakovlev et al., 2008). Signaling pathways involved in short-day regulated dehydrin gene expression remain to be characterized although Welling and Palva (2008) have recently identified four homologues of *CBF* genes in silver birch, named *BpCBF1* to *BpCBF4*, involved in regulation of *BpLti36* gene expression.

Dehydrins which accumulate in bark tissues during winter dormancy often exhibit protective functions. For example, 24 kDa dehydrin from *Betula pubescens*, which accumulates in the vicinity of storage protein bodies and starch-rich amyloplasts under cold, exhibits protective effect on  $\alpha$ -amylase (EC 3.2.1.1.) enzymatic activity *in vitro*. Similarly, dehydrin RcDhn5 from *Rhododendron catawbiense* can protect LDH activity *in vitro* (Peng et al., 2008).

Dehydrins also accumulate in buds during winter dormancy. Yakubov et al. (2005) detected dehydrin protein, PV-DHN, in the outer leaves of inflorescence buds in pistachio (*Pistacia vera*), grown in Negev desert highlands, during December and January. Yakovlev et al. (2008) studied changes in dehydrin gene expression in buds of Norway spruce (*Picea abies*) during bud winter dormancy and subsequent flushing. During bud flushing, expression of some *PaDhn* genes (*PaDhn1*, *PaDhn4.6*, *PaDhn5*, *PaDhn6*, *PaDhn2*, and *PaDhn3*) declines while expression of other *PaDhn* genes (*PaCAP1*, *PaDhn4.2*, and *PaDhn7*) does not change significantly. These results can suggest that dehydrins from the first group may be related to bud winter hardiness while dehydrins from the second group may have general protective functions.

### 10.2.5 DEHYDRINS AND HEAVY-METAL STRESS

Recently, metal ion binding properties have been found in some dehydrins, e.g., in CuCOR15 from *Citrus unshiu* (Hara et al., 2005). Dehydrins may thus function as chelators analogous to metallothioneins and phytochelatins, although different amino acid residues are involved in metal ion binding. Instead of cysteine thiol groups responsible for metal ion binding in metallothioneins and phytochelatins, histidine was reported to bind several metal ions—Co<sup>2+</sup>; Cu<sup>2+</sup>; Fe<sup>2+</sup>; Fe<sup>3+</sup>; Ni<sup>2+</sup>; Zn<sup>2+</sup> in CuCOR15. Recently, some papers showing a positive effect of dehydrin expression on plant heavy-metal tolerance have been published. Xu et al. (2008) found out that the expression of BjDHN2 and BjDHN3 proteins from *Brassica juncea* in transgenic tobacco resulted in plants which accumulated higher concentrations of Cd<sup>2+</sup> and Zn<sup>2+</sup> in their root system with respect to wild-type plants when they were exposed to stress conditions (100  $\mu$ M CdCl<sub>2</sub> or 200  $\mu$ M ZnCl<sub>2</sub> for 10 days) and thus the transformants exhibited higher tolerance to heavy-metal stress. In contrast, *Brassica juncea* plants with inhibited expression of *BjDHN3* (via expression of antisense *BjDHN3* mRNA) revealed increased electrolyte leakage and reduced accumulation of Cd<sup>2+</sup> and Zn<sup>2+</sup>, which

indicates that they were not able to cope with heavy-metal stress. Zhang et al. (2006) have observed increased expression of an SK<sub>n</sub>-type dehydrin PvSR3 transcripts in roots of bean (*Phaseolus vulgaris*) in response to various heavy metals including cadmium and mercury (in the form of 0.2% w/v CdCl<sub>2</sub> or HgCl<sub>2</sub>). Tamás et al. (2006) have observed enhanced expression of *Dhn4* in barley roots in response to aluminum (10 mM AlCl<sub>3</sub> pH 4.0).

Recently, Hara et al. (2009) have reported for CuCOR15 that increased concentrations of Zn<sup>2+</sup> lead to the interaction of this histidine-rich dehydrin with DNA and RNA. They have shown that the CuCOR15 nucleic-acid binding affinity is not sequence-specific; thus, they have hypothesized that binding of CuCOR15 to nucleic acids may have general protective functions under stress conditions.

### 10.2.6 DEHYDRINS AND BIOTIC STRESSES (WOUNDING)

Wounding, i.e., mechanical damage of plant tissues by herbivores or (sucking apparatus of) insects is a common biotic stress that most plants have to face every day. Wounding is associated with cellular damage that leads to water loss. Thus, wounding can also be regarded as a dehydration stress. In wounding stress signaling, salicylic acid (a derivative of 3-hydroxy-3-phenylpropanoic acid), jasmonic acid (a derivative of 12-oxo-*cis,cis*-10,15-phytodienoic acid), and its methyl ester methyl jasmonate play important roles.

For several dehydrins, e.g., CpDHN1 (Y<sub>2</sub>K dehydrin) from *Cicer pinnatifidum* (Bhattarai and Fettig, 2005) or for PgDHN1 (S<sub>8</sub>K<sub>4</sub> dehydrin) from white spruce *Picea glauca* (Richard et al., 2000), induction of gene expression by jasmonic acid and methyl jasmonate was reported. Rouse et al. (1996) carried out a promoter analysis of *A thaliana* cold-inducible K<sub>n</sub>-type dehydrin gene *Lti30* (*Xero 2*) using GUS reporter gene and they concluded that the *Lti30* promoter also revealed induction by wounding (damage of plant roots by forceps) among other treatments. In *Boea crassifolia*, expression of dehydrin *BcDh2* is induced upon wounding via salicylic acid and methyl jasmonate signals (Shen et al., 2004). Recently, Sun et al. (2009) have observed a positive effect of low concentrations of exogenous salicylic acid (up to 0.25 mM) on the expression of drought-inducible dehydrins in barley seedlings subjected to drought stress. In contrast, higher concentrations of salicylic acid (0.25–0.50 mM) have led to the decrease of dehydrin expression under the same growth conditions (water stress). Dehydrins may also play an important role in plant-defense mechanisms. Turco et al. (2004) have reported expression of several dehydrin proteins in drought-tolerant oak species *Quercus ilex* in response to infection with *Phytophthora cinnamomi*.

## 10.3 POSSIBILITIES OF THE USE OF DEHYDRINS FOR IMPROVEMENT OF PLANT TOLERANCE TO STRESS

Along with increasing knowledge of dehydrin protective functions in plant acclimation to various stress factors, attempts to exploit dehydrins for improvement of stress tolerance of economically important plants, especially crops, are becoming quite often recently. These studies include both transgenic techniques (expression of a given dehydrin gene in other organism or simply a modification of a dehydrin promoter sequence in order to enhance dehydrin gene expression) as well as selection of crop varieties with enhanced level of dehydrin expression, which often (but not always) correlates with enhanced level of stress tolerance.

### 10.3.1 TRANSGENIC STUDIES

Several transgenic studies have proven to have a positive effect of dehydrin gene expression and (or) dehydrin protein accumulation on plant stress tolerance. Studies carried out by Saavedra et al. (2006) on the moss *Physcomitrella patens*, which serves as a model organism due to the unique possibility to “knock-out” (make nonfunctional, disrupt) its genes via homologous recombination, have



shown that a *P. patens* knock-out mutant, which has its only dehydrin gene, *PpdhnA*, disrupted, reveals an impaired ability to recover after salt and osmotic stress (treatment either with 0.5 M NaCl or 0.9 M mannitol for 14 days).

In contrast, overexpression of several dehydrin genes under CaMV 35S promoter (constructs pT9 containing *Cor47* and *Rab18* genes and pT10 containing *Lti29* (= *Erd10*) and *Lti30* genes) in *A. thaliana* led to enhanced plant tolerance to cold stress (Puhakainen et al., 2004b). Analogously, Peng et al. (2008) have isolated *RcDHN5* gene (an SK<sub>2</sub> acidic dehydrin) from frost-tolerant *Rhododendron catawbiense* and they have shown that its expression in *A. thaliana* led to the enhancement of frost tolerance. Similarly, Yin et al. (2006) concluded that expression of DHN24 protein from wild potato *Solanum soganandinum* in cucumber (*Cucumis sativus*) led to enhancement of frost tolerance under cold (4°C). Similarly, studies that used a dehydrin transgene expressed in a stress-sensitive plant have reported enhanced tolerance to stress in the transformed plant. For example, Hara et al. (2003) have reported that expression of CuCOR19 from *Citrus unshiu* in tobacco mitochondria led to reduced lipid peroxidation, Houde et al. (2004) have found out that expression of WCOR410 from common wheat in strawberry led to the enhancement of strawberry frost tolerance. Kaye et al. (1998) have found out that tobacco plants expressing spinach CAP85 and CAP160 proteins revealed a lower level of electrolyte leakage after a frost test, which indicates a reduction of freezing injury in the transformants.

Brini et al. (2007b) have observed that expression of DHN-5 protein from durum wheat (*Triticum turgidum* ssp. *durum*) in *A. thaliana* led to the increase in *A. thaliana* salt and osmotic stress tolerance. RoyChoudhury et al. (2007) observed enhanced tolerance to drought and salt stress in tobacco plants transformed with *Rab16A* (= *Rab21*) gene from salt-tolerant indica rice variety Pokkali. The transformed plants exhibited reduced levels of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation as well as lower accumulation of Na<sup>+</sup> and greater accumulation of K<sup>+</sup> when subjected to severe salt stress (200 mM NaCl). Similarly, Cheng et al. (2002) have shown that overexpression of wheat dehydrin PMA80 and wheat LEA I protein PMA1959 enhances rice tolerance to drought and salt stress. Figueras et al. (2004) have reported a positive effect of overexpression of maize Rab17 in *A. thaliana* on osmotic stress tolerance of the transformants. Park et al. (2006) have observed that *Arabidopsis* plants transformed with barley *Dhn3* and *Dhn4* genes stayed green and viable on a GM medium with 500 mM mannitol while *Arabidopsis* wild-type plants died under the same conditions. In contrast, Iturriaga et al. (1992) have not recorded any improvement of drought tolerance determined by ion leakage tests of transgenic tobacco plants expressing several LEA proteins including dehydrins from desiccation-tolerant *Craterostigma plantagineum* when the transformants' leaf discs were subjected to 30% PEG 6,000 solution.

Xu et al. (2008) found out that expression of BjDHN2 and BjDHN3 proteins from *Brassica juncea* in transgenic tobacco resulted in plants which could accumulate higher concentrations of Cd<sup>2+</sup> and Zn<sup>2+</sup> in their root system with respect to wild-type plants when they are exposed to stress conditions (100 µM CdCl<sub>2</sub> or 200 µM ZnCl<sub>2</sub> for 10 days) and thus the transformants exhibit higher tolerance to heavy-metal stress. In contrast, *Brassica juncea* plants with inhibited expression of *BjDHN3* (via expression of antisense *BjDHN3* mRNA) revealed increased electrolyte leakage and reduced accumulation Cd<sup>2+</sup> and Zn<sup>2+</sup>, which indicates that they were not able to cope with heavy-metal stress.

### 10.3.2 DEHYDRINS AS MARKERS OF PLANT STRESS TOLERANCE

A positive effect of dehydrin gene expression or dehydrin protein accumulation on plant stress tolerance has been reported not only by studies using transgenesis, but also by several other studies which compared varieties or cultivars of economically important plants which differ in their level of stress tolerance. Based on these studies, it is becoming evident recently that dehydrins can be used as indirect indicators of plant stress tolerance (about direct and indirect methods of determination of plant stress tolerance, see e.g., Prášil et al., 2007).

Plant tolerance to the effects of a certain stress factor can be associated with allelic variation in dehydrin sequence, which can result in accumulation or absence of a dehydrin protein in a certain plant tissue or organ. Ismail et al. (1999b) studied chilling tolerance during the process of seedling emergence in an extremely chilling-sensitive tropical legume crop *Vigna unguiculata* and they described a presence of DHN1 protein (Y<sub>2</sub>K-type dehydrin of 35 kDa) in seeds of a chilling-tolerant line 1393-2-11, which correlated positively with seedling emergence at 14°C. In contrast, the DHN1 protein was absent in the seeds of a genetically related, but chilling-sensitive line 1393-2-1.

However, the differences in plant stress tolerance are much more often determined by quantitative differences in dehydrin gene expression or dehydrin protein accumulation. It has repeatedly been proven that the rate of dehydrin transcript or protein accumulation positively correlates with the level of plant stress tolerance. In studies dealing with drought stress, Pelah et al. (1997) found a correlation between drought tolerance and accumulation of dehydrin proteins in *Populus popularis*. Park et al. (2006) have found a correlation between *Dhn3* and *Dhn4* transcript accumulation and several characteristics associated with drought tolerance (relative water content RWC, Drought yield index) in a set of Korean barley cultivars. Similarly, Labhili et al. (1995) found a correlation between the level of dehydrin transcript accumulation and drought tolerance in two differently tolerant cultivars of durum wheat (*T. turgidum* ssp. *durum*). Cellier et al. (1998) have compared two lines of sunflower (*Helianthus annuus*) differing in their drought tolerance and found differences in their leaf water potential under the same gravimetric soil water content. When both lines were compared, the tolerant line had a higher leaf water potential under the same gravimetric soil water content and exhibited also a higher level of expression of two dehydrin genes, *HaDhn1* and *HaDhn2*, not only under the same gravimetric soil water content, but also when the plants with the same leaf water potential were compared. Tolerant line also exhibited higher level of *HaDhn2* gene expression after application of exogenous ABA. Analogously, Tabaei-Aghdai et al. (2000) compared dehydrin protein accumulation in drought-tolerant *Agropyron desertorum* and in a less tolerant *Lophopyrum elongatum* under drought stress (6 days without watering) and detected much higher levels of dehydrin polypeptides in the drought-tolerant *A. desertorum* than in *L. elongatum*.

Brini et al. (2007a) studied DHN-5 accumulation in two cultivars of Tunisian durum wheat (*Triticum turgidum* ssp. *durum*) with different level of drought tolerance. They found differences not only in accumulation of DHN-5, but also in the pattern of DHN-5 phosphorylation. The tolerant cultivar showed a high level of DHN-5 phosphorylation while a sensitive cultivar nearly lacked phosphorylated forms of DHN-5. So it can be postulated that different patterns of dehydrin posttranslational modifications, namely, phosphorylation may underlie the differences in cultivar tolerance to stress.

Quantitative differences in dehydrin gene expression and dehydrin protein accumulation with respect to the low-temperature stress (cold and frost) have been studied especially in economically important cereals from tribe *Triticeae*, which are grown in temperate climates. In bread wheat (*T. aestivum*), Houde et al. (1992b) have already described a correlation between accumulation of dehydrin proteins from the WCS120 family and the level of plant-acquired frost tolerance. At the beginning of these studies, only the differences in dehydrin expression between cultivars (lines) with contrasting levels of acquired frost tolerance (i.e., spring cultivars versus winter ones) were studied (e.g., Zhu et al., 2000). However, Vítámvás et al. (2007) have shown that two winter wheat cultivars with a different level of acquired frost tolerance (Mironovskaya 808 and Bezostaya 1) can be distinguished according to the level of accumulation of WCS120 proteins after a 3 week cold treatment (2°C). Kosová et al. (2008) studied accumulation of cold-inducible barley orthologue of the WCS120 protein, DHN5, and found a correlation between DHN5 accumulation and the level of acquired frost tolerance in a set of 21 barley cultivars of different geographical origin and growth habit (facultative, spring, winter) after a 3 week cold treatment. However, Kosová et al. (2010) have shown that the correlation between DHN5 accumulation and acquired frost tolerance or winter survival rate can be obtained only in those barley plants that are still in vegetative phase of their development.

Stress-tolerant and stress-sensitive related plant species or cultivars belonging to one plant species can be distinguished according to level of dehydrin expression not only under stress conditions,

but also under non-stress (or mild stress) growth conditions or in the early phases of stress treatment. This observation was reported by Xiao and Nassuth (2006) on dehydrin transcript *DHN-1* in leaves and buds of two grapevine species *Vitis vinifera* and *Vitis riparia*, which differ in their frost tolerance. The tolerant *Vitis riparia* revealed higher level of *DHN1* under control conditions and also an earlier increase in *DHN1* level under stress conditions than the sensitive *Vitis vinifera*. In *Triticeae*, it has also been shown by several authors that cultivars of wheat or barley with different levels of frost tolerance can be distinguished according to dehydrin (or other *Cor* gene) expression not only at cold temperatures typical for cold acclimation process (5°C–2°C), but also at mild cold temperatures (17°C–9°C). In the first studies published by Crosatti et al. (1995, 1996), spring versus winter cultivars of barley have been distinguished according to the level of expression of chloroplast-located *Cor14b* gene (a *Lea-3* gene). Later, Vágújfalvi et al. (2000) have distinguished highly frost-tolerant winter wheats Albidum and Uljanovka from the less tolerant winter wheat Cheyenne according to *Cor14b* gene expression using the plants cultivated at 18/13°C (day/night) regimen. Holková et al. (2009) quantified the expression of *Wcs120* gene in four winter wheat genotypes by real-time PCR and they were able to distinguish the genotypes with different frost tolerance at 17°C at transcript level. Vítámvás et al. (2010) used dehydrins from the *Wcs120* gene family, and they were able to distinguish 20 cultivars of different frost-tolerant winter wheats grown at 17°C or 9°C at WCS120 protein level. Moreover, the level of accumulation of WCS120 proteins corresponded well with the level of plant winter survival. Thus, it seems possible to use the level of WCS120 accumulation in wheat plants grown under mild cold temperatures (17°C–9°C) as a means for estimation of plant winter survival. These results could enhance the prescreening procedures in the breeding programmes aimed at the improvement of wheat frost tolerance (or winter survival) immensely since the plants can be grown under relatively high temperatures, thus relatively large amounts of plant material can be obtained much faster when compared with plant growth under cold.

## 10.4 CONCLUDING REMARKS

With increasing data from diverse research fields, dehydrins appear to be an amazingly versatile group of LEA proteins presumably due to their intrinsically unstructured character. They exhibit myriads of functions (e.g., chaperone, cryoprotective, antifreeze, radical-scavenging, ion-binding functions) in plant reactions on various stress factors, including drought, high-salinity stress, low-temperature stress, heavy-metal stress, and also some biotic stresses such as wounding. Several studies carried out on both normal (non-transgenic) and transgenic plants have reported a positive effect of dehydrin transcript or protein (expression) accumulation on plant tolerance to various stress factors. Future research studies will surely bring new insights into the functions of these fascinating proteins and we can hope that these studies will significantly contribute to our better understanding of the roles which these stunning proteins play in the conundrum of plant-stress response mechanisms.

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## APPENDIX 10.A: LIST OF SEQUENCED DEHYDRINS

The list of sequenced dehydrin proteins summarizes basic data from NCBI database (<http://www.ncbi.nlm.nih.gov/>) available in July, 2009. The individual plant species are ordered according to their scientific Latin names. Basic protein characteristics including dehydrin structural subgroup

according to the presence of S-, K-, and Y-segments, number of amino acids (aa), protein isoelectric point (pI), and molecular weight (MW) in kilodaltons (kDa) are given. The references which are already given above in the reference list are not repeated in the appendix.

***Aegilops umbellulata*** (Zhuk.) Bowden

*Dhn1* (AM180925.1) (CAJ56055.1) gi 121489505—YSK<sub>2</sub>—150 aa—pI 9.36; MW 15.16 kDa (Rampino et al., 2006).

*Dhn2* (AM180926.1) (CAJ56056.1) gi 121489507—YSK—78 aa—pI 9.16; MW 7.83 kDa (Rampino et al., 2006).

*Dhn3* (AM180927.1) (CAJ56057.1) gi 121489508—YSK<sub>2</sub>—139 aa—pI 8.83; MW 14.35 kDa (Rampino et al., 2006).

*Dhn4* (AM180928.1) (CAJ56058.1) gi 121489511—Kn—96 aa—pI 6.83; MW 9.95 kDa (Rampino et al., 2006).

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***Agropyrum elongatum*** (Host.) Beauv. (tall wheatgrass)

trO64939—K<sub>6</sub>

***Ammopiptanthus mongolicus*** Cheng

trQ6PNN7—KS

***Arabidopsis thaliana*** (L.) Heynh. (thale cress)

*Cor47*—At1g20440—gi 1169280—SK<sub>3</sub>—265 aa—pI 4.44; MW 29.896 kDa (Gilmour et al., 1992).

*Erd10/Lti29/Lti45*—At1g20450—gi 1169277—SK<sub>3</sub>—260 aa—pI 4.85; MW 29.547 kDa (Kiyosue et al., 1994).

*Erd14*—At1g76180—gi 1169278—SK<sub>2</sub>—185 aa—pI 5.19; MW 20.786 kDa (Kiyosue et al., 1994).

*Rab18*—At5g66400—gi 15239373—Y<sub>2</sub>SK<sub>2</sub>—186 aa—pI 7.95; MW 18.463 kDa (Lång and Palva 1992).

*Xero1*—At3g50980—gi 1169340—YSK<sub>2</sub>—128 aa—pI 9.79; MW 13.434 kDa

*Xero2/Lti30*—At3g50970—gi 1169341—K<sub>6</sub>—187 aa—pI 10.07; MW 20.909 kDa (Welin et al., 1994).

*At1g54410*—KS—98 aa—pI 7.23; MW 10.795 kDa

*PAP310*—At2g21490—gi 1592670—Y<sub>2</sub>SK<sub>2</sub>—185 aa—pI 6.9; MW 19.297 kDa

*At4g38410*—SK<sub>2</sub>—163 aa—pI 6.18; MW 18.237 kDa

*At4g39130*—Y<sub>3</sub>K—151 aa—pI 6.59; MW 16.259 kDa

Gene paralogues (local duplications): *Cor47* and *Erd10*; *Xero 1* and *Xero 2*

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***Arachis hypogaea*** L. (peanut)

trQ850G3 gi 194466091 (ACF74276.1) (DQ889511.1)—KS—92 aa—pI 5.21; MW 8.7 kDa (Bi et al. unpublished).

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Bi, Y.P., S.B. Wan, L. Shan, H.T. Zhang, L. Su, X.Q. Quan, and M. Xia. Cloning of some genes expressed in peanut seeds. Unpublished.

***Aster tripolium*** L. (sea starwort)

(AB090885.1) (BAC57962.1) gi 28804513—SK<sub>2</sub>—155 aa—pI 8.83; MW 15.559 kDa (Takeda et al., 2003 unpublished).

## Reference

Takeda M., Y. Uno, M. Kanechi, and N. Inagaki. 2003. Unpublished.

### *Avicennia marina* (Forsk.) Vierh. (gray mangrove)

*AmDHN1* (EU121850) (ABV58321.1) gi 157497149—195 aa—YSK<sub>2</sub>—pI 5.72; MW 19.75 kDa (Mehta et al., 2009).

*AmDHN1a* (EU121851.1) (ABV58322.1) gi 157497151—195 aa—YSK<sub>2</sub>—pI 5.72; MW 19.75 kDa (Mehta et al., 2009).

### *Betula pendula* Roth. (silver birch)

BpLti36—SK<sub>2</sub>

### *Betula pubescens* Ehrh. (pubescent—downy—birch)

*BpuDHN1* (AJ555331) (CAD87733) gi 29603157—YnKn—127 aa—pI 9.16; MW 13.86 kDa (Welling et al., 2004).

*BpuDHN2* (AJ555332) (CAD87734) gi 29565323—SKn—314 aa—pI 5.10; MW 25.27 kDa (Welling et al., 2004).

### *Boea crassifolia* Hemsl.

*Bdn1* (AF190474.2) (AAF01465.2) gi 13992713—SK<sub>3</sub>—252 aa—pI 4.93; MW 29 kDa (Zhao et al., 2001 unpublished).

## References

Zhao, H., H. Liu, Y. Hu, and Z. Lin. 2001. A drought-induced dehydrin like gene from *Boea crassifolia* Hemsl. unpublished.

*Bh2* (AY243045.1) (AAO86690.1) gi 29650475—YSK<sub>2</sub>—133 aa—pI 8.04; MW 14.33 kDa (Shen et al., 2004).

### *Brassica juncea* (L.) Czern. (Indian mustard)

*BjDHN1* (AY130999) (AAN08719) gi 37547535—Y<sub>3</sub>SK<sub>2</sub>—183 aa—pI 6.67; MW 19.2 kDa (Yao et al., 2005).

*BjDHN2* (DQ441470) (ABD95986.1) gi 90654233—SK<sub>2</sub>—243 aa—pI 5.19; MW 27.99 kDa (Xu et al., 2008).

*BjDHN3* (DQ441471.1) (ABD95987.1) gi 90654235—SK<sub>2</sub>—197 aa—pI 5.55; MW 22.05 kDa (Xu et al., 2008).

### *Brassica napus* L. (oilseed rape)

*BnDHN1* (AY303803) (AAQ74768) gi 34539778—Y<sub>3</sub>SK<sub>2</sub>—183 aa—pI 6.67; MW 19.2 kDa (Yao et al., 2005).

*BnERD10* (AY376669) (AAR23753) gi 38564509—S<sub>8</sub>K<sub>2</sub>—271 aa—pI 5.09; MW 31 kDa (Deng et al., 2005).

## Reference

Deng, Z.X., Y.Z. Pang, W.W. Kong, Z.H. Chen, X.L. Wang, X.J. Lin, Y. Pi, X.F.M. Sun, and K.X. Tang. 2005. A novel ABA-dependent dehydrin *ERD10* gene from *Brassica napus*. *DNA sequence* 16:28–35.

### *Brassica oleracea* L. (wild cabbage)

*BOPC34* gi 1154629—SK<sub>2</sub>—199 aa

### *Camellia sinensis* (L.) O. Kuntze. (tea)

(FJ436978.1) (ACJ65691.1) gi 215398978—YSK<sub>3</sub>—201 aa—pI 7.97; MW 21.054 kDa (Li et al., 2008 unpublished).

*Sk2* (GQ228834.1) (ACT10283.1) gi 251736861—Kn—251 aa—pI 5.2; MW 28.68 kDa (Li et al., 2009 unpublished).

(FJ436979.1) (ACJ65692.1) gi 215398980—YSKn—201 aa—pI 7.97; MW 21.054 kDa (Li et al., 2008 unpublished).

(FJ014474.1) (ACH87171.1) gi 198400325—Y<sub>2</sub>SKn—201 aa—pI 7.23; MW 21.112 kDa (Paul et al., 2008 unpublished).

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- Li, Y., W. Deng, Z. Wang, and C. Jiang. 2008. Clone and expression of a dehydrin during desiccation and freezing of tea seeds. Unpublished.
- Li Y., L. Chen, C. Fang, Y. Wang, and C. Jiang. 2009. Construction of SSH cDNA libraries of *Camellia sinensis* under cold stress and cloning of dehydrin genes. Unpublished.
- Paul, A., S. Kumar, and P.S. Ahuja. 2008. Cloning of full length cDNA encoding putative dehydrin from *Camellia sinensis* (L.) O. Kuntze. Unpublished.

### *Camellia oleifera* C.Abel (tea-oil camellia)

(EU856537.1) (ACF72673)—208 aa—pI 6.81; MW 21.5 kDa (Tan et al., unpublished 2008).

## Reference

- Tan, X.F., X.Y. Hu, X.M. Tian, Q. Liu, Q. Luo, and L.S. Xie. 2008. Unpublished.

### *Capsella bursa-pastoris* (L.)Medik. (shepard's purse)

*CbCor29* (DQ090957) (AA084736) gi 68005554—SK<sub>3</sub>—261 aa—pI 4.93; MW 29.4 (Fan and Wang 2006).

## Reference

- Fan, Z.Q. and X.R. Wang. 2006. Isolation and characterization of a novel dehydrin gene from *Capsella bursa-pastoris*. *Mol. Biol.* 40:52–60.

### *Capsicum annuum* L. (pepper)

*CaDhn* (AY225438.1) (AA038853.1) gi 37905913—SK<sub>3</sub>—219 aa—pI 5.41; MW 24.612 kDa (Chung et al., 2003).

## Reference

- Chung, E., S.-Y. Kim, S.Y. Yi, and D. Choi. 2008. *Capsicum annuum* dehydrin, an osmotic-stress gene in hot pepper plants. *Mol. Cells* 15:327–332.

### *Carica papaya* L. (papaya)

tr Q8W267

### *Cicer pinnatifidum* Jaub. and Spach

*CpDhn1* (AY170010) (AAN77521) gi 26245734—Y<sub>2</sub>K—195 aa—pI 5.82; MW 20.4 (Bhattarai and Fettig 2005).

### *Cichorium intybus* L. (cichory)

*Dhn1* (EU791889.1) (ACF15448.1) gi 193161409—SK<sub>4</sub>—262 aa—pI 5.14; MW 29.49 kDa (Mingeot et al., 2009).

*Dhn2* (EU791890.1) (ACF15449.1) gi 193161439—Y<sub>2</sub>K<sub>2</sub>—261 aa—pI 6.33; MW 26.35 kDa (Mingeot et al., 2009).

## Reference

- Mingeot, D., N. Dauchot, P. Van Cutsem, and B. Watillon. 2008. Characterization of two dehydrin genes from *Cichorium intybus* L. *Mol. Biol. Rep.* 36:1995–2001.

### *Citrus clementina* × *Citrus reticulata*

*CrCOR15* (AY327515.1) (AAQ92310) gi 37524017—138 aa—pI 6.54; MW 15.23 kDa (Sánchez-Ballesta et al., 2004).

## Reference

- Sánchez-Ballesta, M.T., M. Rodrigo, M.T. Lafuente, A. Granell, and L. Zacharias. 2004. Dehydrin from citrus, which confers in vitro dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. *J. Agric. Food. Chem.* 52:1950–1957.

***Citrus × paradisi* M. (grapefruit)**

*Cor15* (AY032975) (AAK52077) gi 14031067—K<sub>2</sub>S—137 aa—pI 6.54; MW 15.1 (Porat et al., 2002).  
*cpDHN* (AY160772) (AAN78125) gi 26418686—SK<sub>2</sub>—234 aa—pI 5.62; MW 26.7 (Porat et al., 2004).

***Citrus sinensis* [L.]Osbeck. (orange)**

*csDHN* (AY297793) (AAP56259) gi 31745704—SK—235 aa—pI 7.24; MW 27.2 (Porat et al., 2004).

***Citrus unshiu* Marcov. (Satsuma mandarin)**

*CuCOR15* (AB178479) (BAD97812) gi 63002596—K<sub>2</sub>S—137 aa—pI 6.54; MW 15.2 kDa (Hara et al., 2005).  
*CuCOR19* (AB016809) (BAA74736) gi 4239893—K<sub>3</sub>S—171 aa—pI 6.53; MW 19 kDa (Hara et al., 1999).

***Codonopsis lanceolata* (Siebold and Zucc.) Trautv.**

*Dhn1* (AB126059.1) (BAD18926) gi 47155382—YSK<sub>2</sub>—159 aa—pI 6.87; MW 16.72 kDa (In et al., 2004 unpublished).

**Reference**

In J.G., Yang D.C., Lee B.S., and Lee K. 2004. Unpublished.

***Coffea canephora* var. *Robusta* Pierre ex Froehner (coffee)**

*CcDH1a* (DQ323987.1) (ABC55670) gi 84314116—Y<sub>3</sub>SK<sub>2</sub>—172 aa—pI 7.14; MW 17.78 kDa (Hinniger et al., 2006).  
*CcDH1b* (DQ323988.1) (ABC55671.1) gi 84314118—Y<sub>3</sub>SK<sub>2</sub>—175 aa—pI 7.9; MW 18.08 kDa (Hinniger et al., 2006).  
*CcDH2a* (DQ323989.1) (ABC55672.1) gi 84314120—Y<sub>3</sub>SK<sub>2</sub>—162 aa—pI 6.54; MW 17.4 kDa (Hinniger et al., 2006).  
*CcDH2b* (DQ323990.1) (ABC55673.1) gi 843141122—Y<sub>3</sub>SK<sub>2</sub>—162 aa—pI 6.49; MW 17.37 kDa (Hinniger et al., 2006).  
*CcDH3* (ABC68275.1) gi 8487556 SK<sub>3</sub>.

**Reference**

Hinniger, C., V. Caillet, F. Michoux, M. Ben Amor, S. Tanksley, C. Lin, and J. McCarthy. 2006. Isolation and characterization of cDNA encoding three dehydrins expressed during *Coffea canephora* (Robusta) grain development. *Ann. Bot.* 97:755–765.

***Cornus sericea* L. (red osier dogwood)**

60 kDa dehydrin *Rod60* (AF345988.1) (AAL83426) gi 19032420—YnSKn—566 aa—pI 7.54; MW 60.56 kDa (Sarnighausen et al., 2002 unpublished).  
 48 kDa dehydrin *Rod48* (AF345989.1) (AAL83427) gi 19032422—440 aa—pI 6.25; MW 48.62 kDa (Sarnighausen et al., 2002 unpublished).  
 44 kDa dehydrin *Rod44* (AF345990.1) (AAL83428) gi 19032424—400 aa—pI 6.64; MW 44.24 kDa (Sarnighausen et al., 2002 unpublished).  
 25 kDa dehydrin *Rod25* (AF345991.1) (AAL83429) gi 19032426—236 aa—pI 8.05; MW 25.096 kDa (Sarnighausen et al., 2002 unpublished).

**Reference**

Sarnighausen, E., D.T. Karlson, Y. Zeng, K.G. Raghothama, P.B. Goldsbrough, and E.N. Ashworth. 2002. Characterization of four novel YnSKn type dehydrin-like cDNAs from cold-acclimated red-osier dogwood (*Cornus sericea* L.) xylem. Unpublished.

***Craterostigma plantagineum* Hochst. (blue gem)**

*Dsp14* (P22238) (AAA63612.1) gi 118517—YSK<sub>2</sub>—117 aa—pI 7.92; MW 12.21 kDa (Piatkowski et al., 1990).  
*Dsp16* (P22239) (AAA63613.1) gi 118518—YSK<sub>2</sub>—155 aa—pI 9.70; MW 15.57 kDa (Piatkowski et al., 1990).

***Cupressus sempervirens* L. (cypress)**

*cyp1p114* (FJ379996.1) (ACJ09635.1) gi 210162074—150 aa—pI 9.03; MW 16.65 kDa (Pedron et al., 2009).  
*cyp1p091* (FJ379973.1) (ACJ09612) gi 210162028—SKn—124 aa—pI 4.66; MW 14.18 kDa (Pedron et al., 2009).

- cyplp138* (FJ380020.1) (ACJ09659.1) gi 210162122—Kn—146 aa—pI 4.71; MW 16.11 kDa (Pedron et al., 2009).
- cyplp133* (FJ380015.1) (ACJ09654.1) gi 210162112—Kn—123 aa—pI 5; MW 13.93 kDa (Pedron et al., 2009).
- cyplp116* (FJ379998.1) (ACJ09637.1) gi 210162078—Kn—131 aa—pI 5.03; MW 14.33 kDa (Pedron et al., 2009).
- cyplp093* (FJ379975.1) (ACJ09614.1) gi 210162032—SKn—102 aa—pI 5.62; MW 11.19 kDa (Pedron et al., 2009).
- cyplp086* (FJ379968.1) (ACJ09607.1) gi 210162018—SKn—119 aa—pI 5.82; MW 13.28 kDa (Pedron et al., 2009).
- cyplp071* (FJ237480.1) (ACI87800.1) gi 209778979—SKn—163 aa—pI 5.15; MW 18.37 kDa (Pedron et al., 2009).
- cyplp048* (FJ237457.1) (ACI87777.1) gi 209778933—K—57 aa—pI 4.28; MW 6.28 kDa (Pedron et al., 2009).
- cyplp041* (FJ237450.1) (ACI87770.1) gi 209778919—Kn—102 aa—pI 5.93; MW 12.2 kDa (Pedron et al., 2009).

***Daucus carota* L. (carrot)**

- ECP40 (Embryonic cell protein 40) (Q07322) gi 1706562—YSK<sub>2</sub>—gi 57506540 (BAD86644)—YSK<sub>2</sub>—149 aa—pI 8; MW 15.36 kDa (Shiota et al., 2004).

**Reference**

- Shiota, H., G. Yang, S. Shen, C.H. Eun, K. Watabe, I. Tanaka, and H. Kamada. 2004. Isolation and characterization of six abscisic acid-inducible genes from carrot somatic embryos. *Plant Biotechnol.* 21:309–314.

***Elaeis guineensis* Jacq. (oil palm)**

- (AF236067.1) (AAF60172.1) gi 7330252—YSK<sub>2</sub>—131 aa—pI 7.26; MW 14.1 kDa (Cha and Shah, 2000 unpublished).

**Reference**

- Cha, T.S. and F.H. Shah. 2000. Unpublished.

***Eriobotrya japonica* (Thunb.) Lindl. (loquat; Japanese plum)**

- Dhn1* (ACL01288.1) gi 218750465
- Dhn2* (FJ472836.1) (ACL01289.1) gi 218750467—Kn—128 aa—pI 5.73; MW 14.598 kDa (Xu et al., 2008 unpublished).

**Reference**

- Xu, H.X., J.W. Chen, and M. Xie. 2008. The role of dehydrin in *Eriobotrya japonica* response to cold stress. Unpublished.

***Fagus sylvatica* L. (European beech)**

- FsDhn1* (AJ606474) gi 38343924—YSK<sub>2</sub>—183 aa—20 kDa (Jiménez et al., 2008).

***Ginkgo biloba* L. (maidenhair tree)**

- GbDHN* (AY847265.1) (AAX51663.1) gi 61675972—SK<sub>2</sub>—163 aa—6.61; MW 17.67 kDa (Deng et al., 2006).

**Reference**

- Deng, Z.X., Y.D. Wang, K.J. Jiang, X.F. Liu, W.S. Wu, S. Gao, J. Lin, X.F. Sun, and K.X. Tang. 2006. Molecular cloning and characterization of a novel dehydrin gene from *Ginkgo biloba*. *Biosci. Rep.* 26:203–215.

***Glycine max* L. (soybean)**

- GmPM12* (AF004807) (AAB71225.1) gi 2270990—YSK<sub>2</sub>—166 aa—pI 9.22; MW 17.32 kDa (Hsu et al., 1997 unpublished).
- (AM420412.1) (CAM06618) gi 119709430—Y<sub>2</sub>Kn—242 aa—pI 6.1; MW 25.385 kDa (Chu and Nguyen, 2007 unpublished).
- (AJ583802.1) (CAE47771.1) gi 37495457—Y<sub>2</sub>Kn—242 aa—pI 6.02; MW 25.275 kDa (Nguyen Thi Hong et al., 2003 unpublished).



- (AJ583801.1) (CAE47770.1) gi 37495455—226 aa—pI 5.87; MW 23.73 kDa (Cao Xuan et al., 2003 unpublished).  
 (AJ583800.1) (CAE47769.1) gi 37495453—Y<sub>2</sub>Kn—242 aa—pI 6.26; MW 25.55 kDa (Cao Xuan et al., 2003 unpublished).  
 (AJ583799.1) (CAE47768.1) gi 37495451—Y<sub>2</sub>Kn—226 aa—pI 5.97; MW 23.787 kDa (Tran Thi Phuong et al., 2003 unpublished).  
 SLTI629 (EF190871.1) (ABQ81887.1) gi 148524045—KS—113 aa—pI 6.46; MW 12.534 kDa (Cho et al., 2008 unpublished).  
 Mat1 (L00921.1) (AAA33990) gi 170020—YnKn—243 aa—pI 6.02; MW 25.659 kDa (Chyan et al., 1992 unpublished).  
 Mat9 (U10111.1) (AAA18834) gi 497417—Y<sub>2</sub>Kn—226 aa—pI 6.07; MW 23.717 kDa (Maitra and Cushman, 1994 unpublished).  
 SLTI66 (EF166061.1) (ABO70349.1) gi 134290686—KS—90 aa—pI 8.05; MW 10.068 kDa (Cho et al., 2009 unpublished).  
 GmLea8 (AJ704825.1) (CAG28965) gi 57283637—197 aa—pI 6.08; MW 20.395 kDa (Porcel et al., 2005).  
 GmLea10 (AJ704824.1) (CAG28964) gi 57283635—214 aa—pI 5.97; MW 22.865 kDa (Porcel et al., 2005).

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- Cao Xuan, H., T. Nguyen Dang, L. Tran Thi Phuong, H. Nong Van, and M. Le Thi. 2003. Dehydrin gene sequences of Vietnamese soybean cultivars. Unpublished.  
 Cho, C., E. Chung, K. Kim, and J.-H. Lee. 2008. Low temperature inducible soybean dehydrin SLTI629. Unpublished.  
 Cho, C., E. Chung, K. Kim, and J.-H. Lee. 2009. Soybean SRC1 homolog, SLTI66. Unpublished.  
 Chu M.H. and T.H. Nguyen. 2007. Unpublished.  
 Chyan, Y.-J., R.W. Rinne, L.O. Vodkin, and A.L. Kriz. 1992. Structure and expression of two genes encoding dehydrin-like maturation (MAT) proteins in soybean seeds. Unpublished.  
 Hsu, T.F., F.Y. Tsai, Y.I. Hsing, and T.Y. Chow. 1997. *Glycine max* mRNA for dehydrin. Unpublished.  
 Maitra, N. and J.C. Cushman. 1994. Isolation and expression of a drought-induced cDNA encoding a dehydrin-like (Group 2) protein from soybean leaves. Unpublished.  
 Nguyen Thi Hong, V., H. Cao Xuan, T. Nguyen Dang, L. Tran Thi Phuong, H. Nong Van, and M. Le Thi. 2003. Dehydrin gene sequences of Vietnamese soybean cultivars. Unpublished.  
 Porcel, R., R. Azcón, and J.M. Ruiz-Lozano. 2005. Evaluation of the role of genes encoding for dehydrin proteins (LEA D-11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. *J. Exp. Bot.* 56:1933–1942.  
 Tran Thi Phuong, L., H. Cao Xuan, H. Nong Van, and M. Le Thi. 2003. Dehydrin gene sequences of Vietnamese soybean cultivars. Unpublished.

## *Gossypium hirsutum* L. (upland cotton)

- (M81655.1) (AAA33059.1) gi 167334—YSKn—135 aa—pI 9.49; MW 14.69 kDa (Galau and Close, 1991).  
 (M81654.1) (AAA33058.1) gi 167332—YSK—145 aa—pI 6.79; MW 15.8 kDa (Galau and Close, 1991).  
 Lea D-11 (P09442) gi 118478—YSK—145 aa—pI 6.83; MW 15.81 kDa (Baker et al., 1988)—the first dehydrin described

## Reference

- Galau, G.A. and T.J. Close. 1991. Sequences of the cotton Group 2 LEA/RAB/dehydrin proteins encoded by *Lea3* cDNAs. *Plant Physiol.* 98:1523–1525.

## *Helianthus annuus* L. (common sunflower)

- HaDhn1 (AJ250228.1) (CAC20243.1) gi 12053707—Y<sub>3</sub>SK<sub>2</sub>—253 aa—pI 7.38; MW 25.888 kDa (Ouvrard et al., 1996; Natali et al., 2003; Martinelli and Cavallini, 2007).  
 HaDhn2 (AM051287.1) (CAJ19697.1) gi 121488863—153 aa—pI 5.2; MW 15.83 kDa (Natali et al., 2003).

## References

- Martinelli, F. and A. Cavallini. (2007). Light induces expression of a dehydrin-encoding gene during seedling de-etiolation in sunflower (*Helianthus annuus* L.). *J. Plant Physiol.* 164:263–273.  
 Natali, L., T. Giordani, and A. Cavallini. 2003. Sequence variability of a dehydrin gene within *Helianthus annuus*. *Theor. Appl. Genet.* 106:811–818.  
 Ouvrard, O., F. Cellier, K. Ferrare, D. Tousch, T. Lamaze, J.-M. Dupuis, and F. Casse-Delbart. 1996. Identification and expression of water stress- and abscisic acid-regulated genes in a drought-tolerant sunflower genotype. *Plant Mol. Biol.* 31:819–829.

***Helianthus ciliaris* D.C. (Texas blueweed)**

*Dhn1* (AJ297737.1) (CAC43181) gi 14588999—Y<sub>3</sub>SK<sub>2</sub>—220 aa—pI 6.14; MW 22.588 kDa (Cavallini et al., 2006 unpublished).

**Reference**

Cavallini, A., T. Giordani, and L. Natali. 2006. Evolution of a dehydrin encoding DNA sequence within genus *Helianthus*. Unpublished.

***Helianthus debilis* Nutt. (cucumber-leaf sunflower)**

*cDhn1* (AJ249708.1) (CAC80641.1) gi 27526440—Y<sub>3</sub>SK<sub>2</sub>—244 aa—pI 7.33; MW 24.757 kDa (Cavallini et al., 2006 unpublished).

*dDhn1* (AJ249709.1) (CAC80642.1) gi 27526442—Y<sub>3</sub>SK<sub>2</sub>—246 aa—pI 6.84; MW 24.93 kDa (Cavallini et al., 2006 unpublished).

*sDhn1* (AJ249710.1) (CAC80643.1) gi 27526444—Y<sub>3</sub>SK<sub>2</sub>—242 aa—pI 6.43; MW 24.47 kDa (Cavallini et al., 2006 unpublished).

***Helianthus hirsutus* (Torr. & Gray) (hairy sunflower)**

*Dhn1* (AJ250145.1) (CAC80706) gi 27526446—Y<sub>3</sub>SK<sub>2</sub>—240 aa—pI 6.38; MW 24.5 kDa (Cavallini et al., 2006 unpublished).

***Helianthus maximiliani* Schrad. (Maximilian sunflower)**

*Dhn1* (AJ250149.1) (CAC80709.1) gi 27526454—Y<sub>3</sub>SK<sub>2</sub>—228 aa—pI 6.79; MW 23.45 kDa (Cavallini et al., 2006 unpublished).

***Helianthus mollis* Lam. (ashy or downy sunflower)**

*Dhn1* (AJ250146.1) (CAC80707.1) gi 27526448—Y<sub>3</sub>SK<sub>2</sub>—241 aa—pI 6.63; MW 24.623 kDa (Cavallini et al., 2006 unpublished).

***Helianthus neglectus* Heiser (neglected sunflower)**

*Dhn1* (AJ250150.1) (CAC807010.1) gi 27526456—Y<sub>3</sub>SK<sub>2</sub>—246 aa—pI 7.33; MW 25.03 kDa (Cavallini et al., 2006 unpublished).

**Reference**

Cavallini A., Giordani T., Natali L., and Pardini A. unpublished.

***Helianthus niveus* (Benth.) Brandeg. (showy sunflower)**

*Dhn1* (AJ250147.1) (CAC80708.1) gi 27526450—Y<sub>3</sub>SK<sub>2</sub>—246 aa—pI 6.54; MW 25 kDa (Cavallini et al., 2006 unpublished).

***Helianthus petiolaris* Nutt. (prairie sunflower)**

*Dhn1* (AJ250152.1) (CAC80712) gi 27526460—Y<sub>3</sub>SK<sub>2</sub>—240 aa—pI 6.92; MW 24.09 kDa (Cavallini et al., 2006 unpublished).

***Helianthus praecox* Engelm. & Gray (Texas sunflower)**

*Dhn1* (AJ250126.1) (CAC80716.1) gi 18073230—Y<sub>3</sub>SK<sub>2</sub>—246 aa—pI 6.84; MW 24.945 kDa (Cavallini et al., 2006 unpublished).

***Helianthus tuberosus* L. (Jerusalem artichoke; topinambour)**

*Dhn1* (AJ250148.1) (CAC80714.1) gi 27526452—Y<sub>3</sub>SK<sub>2</sub>—240 aa—pI 6.38; MW 24.58 kDa (Cavallini et al., 2006 unpublished).

***Hippophae rhamnoides* ssp. *sinensis* L. (sea buckthorn)**

(AY332226.1) (AAP94627.1) gi 33114013—YSK<sub>2</sub>—160 aa—pI 9.25; MW 16.786 kDa (Tang and Pappinen, 2003 unpublished).

**Reference**

Tang, X. and A. Pappinen. 2003. Dehydrin proteins and their transcripts in association with freezing tolerance of sea buckthorn (*Hippophae rhamnoides*) origins and hybrid. Unpublished.

***Hordeum vulgare* L. (barley)**

*Dhn1* (AF181451) (AAF01690)—YSK<sub>2</sub>—139 aa—pI 8.81; MW 14.2 kDa (Choi et al., 1999).

*Dhn2* (AF181452) (AAF01690)—YSK<sub>2</sub>—141 aa—pI 8.81; MW 14.4 kDa (Choi et al., 1999).

- Dhn3* (AF181453) (AAF01691)—YSK<sub>2</sub>—155 aa—pI 8.07; MW 15.7 kDa (Choi et al., 1999).  
*Dhn4* (AF181454) (AAF01692)—YSK<sub>2</sub>—205 aa—pI 8.04; MW 20.7 kDa (Choi et al., 1999).  
*Dhn5* (AF181455) (AAF01692)—K<sub>9</sub>—575 aa—pI 6.65; MW 58.5 kDa (Close et al., 1995).  
*Dhn6* (AF181456) (AAF01694)—YSK<sub>3</sub>—486 aa—pI 8.80; MW 46.15 kDa (Choi et al., 1999).  
*Dhn7* (AF181457) (AAF01693)—YSK<sub>2</sub>—181 aa—pI 9.10; MW 18.1 kDa (Choi et al., 1999).  
*Dhn8* (AF181458) (AAF01696)—SK<sub>3</sub>—255 aa—pI 5.21; MW 27.7 kDa (Choi et al., 1999).  
*Dhn9* (AF181459) (AAF01697)—YSK<sub>2</sub>—146 aa—pI 9.52; MW 15.1 kDa (Choi et al., 1999).  
*Dhn10* (AF043095) (AAD02261)—YSK<sub>3</sub>—295 aa—pI 9.67; MW 29.15 kDa (Choi et al., 1999).  
*Dhn11* (AF043086) (AAD02252)—Y<sub>2</sub>SK<sub>2</sub>—232 aa—pI 6.26; MW 23.5 kDa (Choi et al., 1999).  
*Dhn12* (AF155129) (AAD38400)—YSK<sub>2</sub>—141 aa—pI 6.59; MW 14.2 kDa (Choi and Close, 2000).  
*Dhn13* (AY681974) (AAT81473)—KS—107 aa—pI 6.84; MW 12 kDa (Rodriguez et al., 2005).

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 Rodriguez, E.M., J.T. Svensson, M. Malatrasi, D.-W. Choi, and T.J. Close. 2005. Barley *Dhn13* encodes a KS-type dehydrin with constitutive and stress responsive expression. *Theor. Appl. Genet.* 110:852–858.

### *Hyacinthus orientalis* L. (hyacinth)

(AY389583.1) (AAT08674.1) gi 47026904—202 aa—pI 5.47; MW 21.76 kDa (Fan et al., 2004 unpublished).

## Reference

- Fan, J.H., Y. Ma, and X.S. Zhang. 2004. Unpublished.

### *Lactuca sativa* L. (lettuce)

*LsLeal* (AJ704826.1) (CAG28966) gi 57283639—YK—113 aa—pI 9.07; MW 12.29 kDa (Porcel et al., 2005).

## Reference

- Porcel, R., R. Azcón, and J.M. Ruiz-Lozano. 2005. Evaluation of the role of genes encoding for dehydrin proteins (LEA D-11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. *J. Exp. Bot.* 56:1933–1942.

### *Lavatera thuringiaca* L. (mallow)

*LtCor18* (AF044584.1) (AAC02689.1) gi 2865523—SK<sub>2</sub>—165 aa—pI 5.05; MW 18.62 kDa (Vazquez-Tello A., 1998 unpublished).

## Reference

- Vazquez-Tello, A. 1998. Genetics of freezing tolerance. Cloning and characterization of cold regulated genes of *Lavatera thuringiaca* (*Malvaceae*). Unpublished.

### *Lemna gibba* L. (fat duckweed)

*Npr1* gi 542105 tr Q40186—SK—151 aa

### *Lindernia brevidens* Skan

*Lb6-19* (EF429273.1) (ABO031098.1) gi 129562715—SK<sub>2</sub>—144 aa—pI 8.9; MW 14.44 kDa (Phillips et al., 2008).

## Reference

- Phillips, J.R., E. Fischer, M. Baron, N. van der Dries, F. Facchinelli, M. Kutzer, R. Rahmzadeh, D. Remus, and D. Bartels. 2008. *Lindernia brevidens*: A novel desiccation-tolerant vascular plant, endemic to ancient tropical rainforests. *Plant J.* 54:938–948.

### *Lophopyrum elongatum* (Host.) Á Löve (tall wheatgrass)

*ESI18-2* (AF031247.1) (AAC05921.1) gi 2970211—Kn—413 aa—pI 6.45; MW 41.66 kDa (Gulick and Dvorak, 1992).

*ESI18-3* (AF031248.1) (AAC05922.1) gi 2970213—YSK<sub>2</sub>—156 aa—pI 8.03; MW 15.91 kDa (Gulick and Dvorak, 1992).

*ESI18-4* (AF0312249.1) (AAC05923.1) gi 2970215—K<sub>3</sub>—124 aa—pI 6.58; MW 12.76 kDa (Gulick and Dvorak, 1992).

*ESI18-5* (AF0312250.1) (AAC05924.1) gi 2970217—K<sub>3</sub>—124 aa—pI 6.08; MW 12.73 kDa (Gulick and Dvorak, 1992).

## Reference

Gulick, P.J. and J. Dvorak. 1992. Coordinate gene response to salt stress in *Lophopyrum elongatum*. *Plant Physiol.* 100:1384–1388.

***Lupinus albus* L.** (white lupine)

*Rab16-type* (AY427790.2) (AAT06600.2) gi 118603318—SK<sub>3</sub>—219 aa—pI 5.39; MW 24.83 kDa (Pinheiro et al., 2008).

## Reference

Pinheiro, C., M.H. Cruz de Cavalho, D. Bartels, C. Pinto Ricardo, and M. Chaves. 2008. Dehydrins in *Lupinus albus*: Pattern of protein accumulation in response to drought. *Funct. Plant Biol.* 35:85–91.

***Lycopersicon esculentum* L.** (tomato)

*TAS14* (U26423.1) (AAC49618.1) gi 1794186—YSK<sub>2</sub>—130 aa—pI 6.06; MW 13.95 kDa (Godoy et al., 1990).

## Reference

Godoy, J.A., J.M. Pardo, and J.A. Pintor-Toro. 1990. A tomato cDNA inducible by salt stress and abscisic acid: Nucleotide sequence and expression pattern. *Plant Mol. Biol.* 15:695–705.

***Malus × domestica* L.** (apple tree)

*MaDhn* (DQ660905.1) (ABG56268.1) gi 110238587—Y<sub>2</sub>SK<sub>4</sub>—229 aa—pI 6.61; MW 24.19 kDa (Garcia-Banuelos et al., 2007 unpublished).

## Reference

Garcia-Banuelos, M.L., L. Vazquez-Moreno, A.A. Gardea, and J.J. Winzerling. 2007. Characterization and expression of a dehydrin gene in apple tree (*Malus domestica*). Unpublished.

***Medicago falcata* L.** (alfalfa)

*Cas18*—gi 484641—K<sub>3</sub>—197 aa (Wolfrain et al., 1993).

***Medicago sativa* L.** (alfalfa)

*Cas15a* gi 289120—K<sub>2</sub>S—136 aa (Monroy et al., 1993).

*Cas15b* gi 289121—K<sub>2</sub>S—136 aa (Monroy et al., 1993).

***Nicotiana tabacum* L.** (common tobacco)

*NtERD10a* (AB049335.1) (BAD13497.1) gi 46020010—31 aa—pI 8.43; MW 3.5 kDa (Kasuga et al., 2004).

*NtERD10b* (AB049336.1) (BAD13498.1) gi 46020012—Y<sub>2</sub>SK<sub>2</sub>—169 aa—pI 6.81; MW 17.77 kDa (Kasuga et al., 2004).

*NtERD10c* (AB049337.1) (BAD13499.1) gi 46020014—Y<sub>2</sub>SK<sub>2</sub>—208 aa—pI 5.62; MW 23.62 kDa (Kasuga et al., 2004).

*NtERD10d* (AB049338.1) (BAD13500.1) gi 46020016—98 aa—pI 5.25; MW 10.956 kDa (Kasuga et al., 2004).

## Reference

Kasuga, M., S. Miura, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2004. A combination of the *Arabidopsis* *DREB1A* gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* 45:346–350.

***Oryza sativa* L.** (rice)—8 dehydrin genes

*OsLEA22* (*RAB25*) (P30287) gi 85681933—YSK<sub>2</sub>—228 aa—pI 6.64; MW 23.236 kDa (Kusano et al., 1992).

## References

- Kusano T., K. Aguan, M. Abe, and K. Sugawara. 1992. Nucleotide sequence of a rice *rab16* homologue. *Plant Mol. Biol.* 18 (1):127–129. Source: *Oryza sativa* Japonica group cv. Akitakomachi.
- OsDhn1* (AY786415.1) (AAV49032.1) gi 55274278—SK<sub>3</sub>—290 aa—pI 5.68; MW 30.922 kDa (Lee et al., 2004 unpublished).
- OsLEA23*.
- OsLEA24*.
- OsLEA25*.
- OsLEA26 (RAB16B)* (A2ZDX8) gi 152013503—YSK<sub>2</sub>—164 aa—pI 9.42; MW 16.56 kDa (Yamaguchi-Shinozaki et al., 1990).
- OsLEA27 (RAB16C)* (A2ZDX6) gi 152013504—YnSKn—164 aa—pI 9.25; MW 16.73 kDa (Yamaguchi-Shinozaki et al., 1990).
- OsLEA28 (RAB16D)* (A2ZDX4) gi 152013505—YnSKn—151 aa—pI 9.13; MW 15.55 kDa (Yamaguchi-Shinozaki et al., 1990).
- OsLEA29 (RAB21 = RAB16A)* (A2ZDX9) gi 152013509—YSK<sub>2</sub>—172 aa—pI 9.19; MW 17.325 kDa (Mundy and Chua 1988)—source: *Oryza sativa* Indica group.

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- Lee, S.-C., M.-Y. Lee, S.-J. Kim, S.-H. Jun, G. An, and S.-R. Kim. 2004. Characterization of a stress-inducible dehydrin gene, *OsDhn1*, from rice (*Oryza sativa* L.). Unpublished.
- Yamaguchi-Shinozaki, K., J. Mundy, and N.-H. Chua. 1990. Four tightly linked *rab* genes are differentially expressed in rice. *Plant Mol. Biol.* 14:29–39.

### *Paeonia suffruticosa* Andrews (tree peony)

- dehydrin 1-like* (EU091154.1) (ABW86619.1) gi 158958099—K—49 aa—pI 9.78; MW 5.11 kDa (Huang et al., 2008).

## Reference

- Huang, X., G.S. Zheng, S.I. Dai, and S.P. Gai. 2008. Identification of differentially expressed genes associated with bud dormancy release in tree peony (*Paeonia suffruticosa*) by expression subtractive hybridization. *For. Stud. China* 10:88–94.

### *Panax ginseng* C.A. Meyer (Korean ginseng)

- PgDhn1* (DQ487106.1) (ABF48474.1) gi 94962317—YSK<sub>3</sub>—197 aa—pI 8.46; MW 20.578 kDa (Ha et al., 2006).
- PgDhn2* (DQ4847107.1) (ABF48475.1) gi 94962319—KS—101 aa—pI 7.12; MW 10.774 kDa (Ha et al., 2006).
- PgDhn3* (DQ4847108.1) (ABF48476.1) gi 94962321—KS—96 aa—pI 6.75; MW 10.164 kDa (Ha et al., 2006).
- PgDhn4* (DQ4847109.1) (ABF48477.1) gi 94962323—SK<sub>3</sub>—218 aa—pI 4.8; MW 24.505 kDa (Ha et al., 2006).
- PgDhn5* (DQ4847110.1) (ABF48478.1) gi 94962325—YSK<sub>2</sub>—174 aa—pI 9.58; MW 18.15 kDa (Ha et al., 2006).
- PgDhn6* (DQ4847111.1) (ABF48479.1) gi 94962327—SK<sub>3</sub>—168 aa—pI 6.1; MW 16.99 kDa (Ha et al., 2006).
- PgDhn7* (DQ4847112.1) (ABF48480.1) gi 94962329—YSK<sub>3</sub>—196 aa—pI 6.67; MW 20.385 kDa (Ha et al., 2006).
- PgDhn8* (DQ4847113.1) (ABF48481.1) gi 94962331—SK<sub>3</sub>—229 aa—pI 6.07; MW 25.928 kDa (Ha et al., 2006).
- PgDhn9* (DQ4847114.1) (ABF48482.1) gi 94962333—SK<sub>2</sub>—82 aa—pI 9.6; MW 8.78 kDa (Ha et al., 2006).

## Reference

- Ha, Y.I., J.-M. Lim, S.M. Ko, J.R. Liu, and D.-W. Choi. 2006. Sequence variability and expression characteristics of the Ginseng (*Panax ginseng* C.A. Meyer) dehydrin gene family. *J. Plant Biol.* 49:205–211.

### *Paspalum juergensii* Hack.

- DHN1* (EU257860.1) (ABX58143.1) gi 161138352—SK—73 aa—pI 11.46; MW 7.79 kDa (Speranza and Bonnacarrere, 2007 unpublished).

### *Paspalum quadrifarium* Lam. (tussock paspalum)

- DHN1* (EU257859.1) (ABX58142.1) gi 161138350—SK—73 aa—pI 11.46; MW 7.79 kDa (Speranza and Bonnacarrere, 2007 unpublished).

## Reference

Speranza, P.R. and V. Bonnacerrere. 2007. Unpublished.

***Pennisetum glaucum*** (L.) R. Br. (pearl millet)  
gi 56412211—YSK<sub>2</sub>

***Phaseolus vulgaris*** L. (common bean)  
PvSR3 (U54703.1) (AAB00554.1) gi 1326161- SK<sub>2</sub>—202 aa—pI 5.32; MW 22.97 kDa (Zhang et al., 2006).

***Phoenix dactylifera*** L. (date palm)  
(DQ399792.1) (ABD66071.1) gi 89275335—117 aa—pI 6.91; MW 12.27 kDa (Aberlenc-Bertossi et al., 2006 unpublished).

## Reference

Aberlenc-Bertossi, F., Y. Duval, and A. Borgel. 2006. Unpublished.

***Physcomitrella patens*** (Hedw.) Bruch & Schimp.

PpDHNA (AY365466.1) (AAR13080) gi 38176433—Y<sub>11</sub>K—554 aa—pI 5.61; MW 59.16 kDa (Saavedra et al., 2006).

***Picea abies*** (L.)Karst. (Norway spruce)

PaDhn1 (AY961924.2) (AAX92687.1) gi 62642096 -K<sub>2</sub>—84 aa—pI 9.1; MW 9.15 kDa (Yakovlev et al., 2006).

PaDhn2.1 (AY961926.1) (AAX92689.1) gi 62642100—SK—171 aa—pI 7.11; MW 18.5 kDa (Yakovlev et al., 2006).

PaDhn3—SK<sub>2</sub> (Yakovlev et al., 2008).

PaDhn4.1 (EF507858) SK<sub>2</sub>.

PaDhn4.2 (EF507859) SK<sub>2</sub>.

PaDhn4.3 (EF507860.1) (ABS58629.1) gi 154103628—SK<sub>2</sub>—158 aa—pI 6.91; MW 17.45 kDa (Yakovlev et al., 2008).

PaDhn4.4 (EF522169) SK<sub>2</sub>.

PaDhn4.5 (EF522170) SK<sub>2</sub>.

PaDhn4.6 (EF522165) SK<sub>2</sub>.

PaDhn5 (EF522171)—SK<sub>4</sub> (Yakovlev et al., 2008).

PaDhn6 (EF522172)—K<sub>3</sub> (Yakovlev et al., 2008).

PaDhn7 (EF522167)—K<sub>2</sub> (Yakovlev et al., 2008).

PaCAP1.1 (EF507861) (ABS58630.1) gi 154103630—S<sub>2</sub>K<sub>6</sub>—183 aa—pI 9.84; MW 19.83 kDa (Yakovlev et al., 2008).

PaCAP1.2 (EF507862) (ABS58631.1) gi 154103632—S<sub>1</sub>K<sub>6</sub>—205 aa—pI 9.88; MW 22.1 kDa (Yakovlev et al., 2008).

## Reference

Yakovlev, I.A., C.-G. Fossdal, Ø. Johnsen, O. Juntilla, and T. Skrøppa. 2006. Analysis of gene expression during bud burst initiation in Norway spruce via ESTs from subtracted cDNA libraries. *Tree Genet. Genomes* 2:39–52.

***Picea glauca*** (Moench.)Voss. (white spruce)

PgDhn1 (AF109916) (AAD28175) gi 4704603—S<sub>8</sub>K<sub>4</sub>—245 aa—pI 6.9; MW 27 kDa (Richard et al., 2000).

***Picea sitchensis*** Bongard (Sitka spruce)

(EF086087.1) (ABK25374.1) gi 116789764—Kn—88 aa—pI 8.84; MW 9.68 kDa (Raplh et al., 2008).

(EF082276.1) (ABK21645.1) gi 116780352—Kn—88 aa—pI 8.98; MW 9.714 kDa (Raplh et al., 2008).

(BT071712.1) (ACN41162.1) gi 224286919—Kn—88 aa—pI 8.84; MW 9.684 kDa (Raplh et al., 2008).

## Reference

Ralph, S.G., R. Kirkpatrick, H.J.E. Chun, D. Palmquist, B. Wynhoven, N. Kolosova, N. Cooper et al. 2008. A conifer genomic resource of 200,000 spruce (*Picea* spp.) ESTs and 6,464 high-quality, sequence-finished full-length cDNAs from Sitka spruce (*Picea sitchensis*). *BMC Genomics* 9:484.

***Pinus banksiana* Lamb.**

*Dhn1* (FJ415434.1) (ACL27820.1) gi 219562721—SKn—163 aa—pI 6.21; MW 18.47 kDa (Palme et al., 2009).

*Dhn2* (FJ415438.1) (ACL27824.1) gi 219562729—Kn—73 aa—pI 9.07; MW 7.99 kDa (Palme et al., 2009).

*Dhn7* (FJ415449.1) (ACL27835.1) gi 219562751—Kn—92 aa—pI 8.68; MW 9.893 kDa (Palme et al., 2009).

**Reference**

Palme, A.E., T. Pyhajarvi, W. Wachowiak, and O. Savolainen. 2009. Selection on a nuclear genes in a *Pinus* phylogeny. *Mol. Biol. Evol.* 26:893–905.

*Dhn9* (FJ415455.1) (ACL27841.1) gi 219562763—SKn—211 aa—pI 6.44; MW 22.625 kDa (Palme et al., 2009).

***Pinus contorta* Dougl. (lodgepole pine)**

*Dhn1* (FJ415433.1) (ACL27819.1) gi 219562719—SKn—163 aa—pI 6.21; MW 18.47 kDa (Palme et al., 2009).

*Dhn2* (FJ415437.1) (ACL27823.1) gi 219562727—Kn—73 aa—pI 9.27; MW 8.038 kDa (Palme et al., 2009).

*Dhn3* (FJ415441.1) (ACL27827.1) gi 219562735—Kn—89 aa—pI 8.6; MW 9.727 kDa (Palme et al., 2009).

*Dhn7* (FJ415448.1) (ACL27834.1) gi 219562749—Kn—92 aa—pI 8.68; MW 9.892 kDa (Palme et al., 2009).

*Dhn9* (FJ415454.1) (ACL27840.1) gi 219562761—SKn—211 aa—pI 6.44; MW 22.67 kDa (Palme et al., 2009).

***Pinus densata* (Masters) Shaw**

*DEH1* (DQ232932.1) (ABB54910.1) gi 81026046—Kn—132 aa—pI 9.16; MW 14.059 kDa (Ma et al., 2006).

**Reference**

Ma, X.F., A.E. Szmidt, and X.R. Wang. 2006. Genetic structure and evolutionary history of a diploid hybrid pine *Pinus densata* inferred from the nucleotide variation at seven gene loci. *Mol. Biol. Evol.* 23:807–816.

***Pinus elliotii* Engelm. (slash pine)**

*Dhn2* (EU349119.1) (ACA51879.1) gi 1692249332—SKn—146 aa—pI 8.74; MW 15.535 kDa (Ersoz et al., 2008 unpublished).

***Pinus halepensis* Mill.**

*Dhn1* (FJ588496.1) (AC057100.1) gi 226440356—SKn—120 aa—pI 9.36; MW 12.719 kDa (Grivet et al., 2008 unpublished).

**Reference**

Grivet, D., F. Sebastiani, S.C. González-Martínez, and G.G. Vendramin. 2008. Demographic and adaptive consequences of long-range colonization in pine. Unpublished.

***Pinus lambertiana* Douglas (sugar pine)**

*Dhn3* (FJ415445.1) (ACL27831.1) gi 219562743—Kn—89 aa—pI 8.6; MW 9.727 kDa (Palme et al., 2009).

***Pinus nigra* Arnold**

*Dhn1* (FJ4154) (ACL27816.1) gi 219562713—SKn—167 aa—pI 6.93; MW 18.852 kDa (Palme et al., 2009).

*Dhn3* (FJ415442.1) (ACL27828.1) gi 219562737—Kn—89 aa—pI 8.6; MW 9.727 kDa (Palme et al., 2009).

*Dhn9* (FJ415452.1) (ACL27838.1) gi 219562757—SKn—210 aa—pI 6.96; MW 22.576 kDa (Palme et al., 2009).

***Pinus peuce* Griseb. (Macedonian pine)**

*Dhn3* (FJ415444.1) (ACL27830.1) gi 219562741—Kn—89 aa—pI 8.6; MW 9.727 kDa (Palme et al., 2009).

***Pinus pinaster* (Ten.) Lindl. & Gordon**

*Dhn1* (FJ415432.1) (ACL27818.1) gi 219562717—SKn—174 aa—pI 6.01; MW 19.899 kDa (Palme et al., 2009).

*Dhn2* (FJ415436.1) (ACL27822.1) gi 219562725—Kn—105 aa—pI 9.22; MW 11.896 kDa (Palme et al., 2009).

*Dhn3* (FJ415440.1) (ACL27826.1) gi 219562733—Kn—89 aa—pI 8.6; MW 9.727 kDa (Palme et al., 2009).  
*Dhn7* (FJ415446.1) (ACL27832.1) gi 219562745—Kn—92 aa—pI 8.36; MW 9.927 kDa (Palme et al., 2009).  
*Dhn9* (FJ415451.1) (ACL27837.1) gi 219562755—SKn—211 aa—pI 8.53; MW 23.648 kDa (Palme et al., 2009).

***Pinus ponderosa* Engelm.**

*Dhn1* (FJ415435.1) (ACL27821.1) gi 219562723—SKn—164 aa—pI 6.41; MW 18.56 kDa (Palme et al., 2009).  
*Dhn2* (FJ415439.1) (ACL27825.1) gi 219562731—Kn—105 aa—pI 9.01; MW 11.61 kDa (Palme et al., 2009).  
*Dhn7* (FJ415450.1) (ACL27836.1) gi 219562753—Kn—92 aa—pI 8.9; MW 9.96 kDa (Palme et al., 2009).  
*Dhn9* (FJ415456.1) (ACL27842.1) gi 219562765—SKn—211 aa—pI 6.58; MW 22.737 kDa (Palme et al., 2009).

***Pinus radiata* D. Don. (Monterey pine)**

*Dhn 2* (EU394115.1)(ACA51875.1) gi 169249324—SK—91 aa—pI 9.52; MW 9.53 kDa (Ersoz et al., 2008 unpublished).

**Reference**

Ersoz, E.S., M.H. Wright, S.C. Gonzalez-Martinez, C.H. Langley, and D.B. Neale. 2008. Patterns of nucleotide diversity and selective constraint in the loblolly pine genome. Unpublished.

***Pinus resinosa* Soland. (red pine)**

*Dhn1* (FJ415431.1) (ACL27817.1) gi 219562715—SKn—167 aa—pI 6.93; MW 18.852 kDa (Palme et al., 2009).  
*Dhn3* (FJ415443.1) (ACL27829.1) gi 219562739—Kn—89 aa—pI 8.68; MW 9.74 kDa (Palme et al., 2009).  
*Dhn7* (FJ415447.1) (ACL27833.1) gi 219562747—Kn—92 aa—pI 8.9; MW 9.92 kDa (Palme et al., 2009).  
*Dhn9* (FJ415453.1) (ACL27839.1) gi 219562759—SKn—212 aa—pI 6.54; MW 22.856 kDa (Palme et al., 2009).

***Pinus sylvestris* L. (Scots pine)**

*Dhn1* (FJ201260.1) (ACJ37596.1) gi 212724322—SK<sub>4</sub>—182 aa—pI 8.42; MW 20.478 kDa (Wachowiak et al., 2009).  
*Dhn2* (FJ201300.1) (ACJ37636.1) gi 212724402—SKn—87 aa—pI 9.40; MW 9.693 kDa (Wachowiak et al., 2009).  
*Dhn3* (FJ201336.1) (ACJ37672.1) gi 212724474—K<sub>2</sub>—89 aa—pI 8.31; MW 9.727 kDa (Wachowiak et al., 2009).  
*Dhn4* (FJ201372.1) (ACJ37708.1) gi 212724546—K<sub>2</sub>—70 aa—pI 8.60; MW 7.687 kDa (Wachowiak et al., 2009).  
*Dhn5* (FJ201412.1) (ACJ37540.1) gi 212724179—K<sub>2</sub>—78 aa—pI 7.86; MW 8.60 kDa (Wachowiak et al., 2009).  
*Dhn7* (FJ201452.1) (ACJ37748.1) gi 212724626—K<sub>2</sub>—88 aa—pI 8.82; MW 9.545 kDa (Wachowiak et al., 2009).  
*Dhn9* (FJ201487.1) (ACJ37783.1) gi 212724696—SKn—210 aa—pI 6.57; MW 22.662 kDa (Wachowiak et al., 2009).

***Pinus tabulaeformis* Carrière**

*DEH1* (DQ233069.1) (ABB55046.1) gi 81031802—Kn—132 aa—pI 9.15; MW 14 kDa (Ma et al., 2006).

**Reference**

Ma, X.F., A.E. Szmids, and X.R. Wang. 2006. Genetic structure and evolutionary history of a diploid hybrid pine *Pinus densata* inferred from the nucleotide variation at seven gene loci. *Mol. Biol. Evol.* 23:807–816.

***Pinus taeda* L. (loblolly pine)**

*Dhn1* (AY867598) (AAW59259.1) gi 57908263—SKn—176 aa—pI 7.04; MW 18.744 kDa (González-Martínez et al., 2006).  
*Dhn2* (AY867503.1) (AAW59164.1) gi 57908057—SK—143 aa—pI 8.43; MW 15.2 kDa (González-Martínez et al., 2006).



## Reference

González-Martínez, S.C., E. Ersoz, G.R. Brown, N.C. Wheeler, and D.B. Neale. 2006. DNA sequence variation and selection of tag single-nucleotide polymorphisms at candidate genes for drought stress response in *Pinus taeda* L. *Genetics* 172:1915–1926.

### *Pinus yunnanensis* Franchet (Yunnan pine)

*DEH1* (DQ233157.1) (ABB55134.1) gi 81035465—K<sub>n</sub>—132 aa—pI 9.15; MW 14 kDa (Ma et al., 2006).

### *Pistacia vera* L. (pistachio)

*PV-dhn* (Y07600) (CAC34554) gi 13375171—K<sub>5</sub>—230 aa—pI 6.76; MW 25.87 (Yakubov et al., 2005).

### *Pisum sativum* L. (garden pea)

*PsDHN1* (P28639) gi 118586—197 aa—pI 5.96; MW 20.44 kDa (Robertson and Chandler, 1992).

*PsDHN2* (P28640) gi 118587—232 aa—pI 6.35; MW 24.49 kDa (Robertson and Chandler, 1992).

*PsDHN3* (P28641) gi 118588—232 aa—pI 6.03; MW 23.947 kDa (Robertson and Chandler, 1992).

## Reference

Robertson, M. and P.M. Chandler. 1992. Pea dehydrins: Identification, characterization and expression. *Plant Mol. Biol.* 19:1031–1044.

### *Plantago maior* L. (common plantain)

*Dhn1* (AJ844000.1) (CAH59415.1) gi 53748473—SK<sub>2</sub>—229 aa—pI 4.95; MW 25.895 kDa (Pommerrenig et al., 2006).

## Reference

Pommerrenig, B., I. Barth, M. Niedermeier, S. Kopp, J. Schmid, R.A. Dwyer, R.J. McNair, F. Klebl, and N. Bauer. 2006. Common plantain. A collection of expressed sequence tags from vascular tissue and a simple and efficient transformation method. *Plant Physiol.* 142:1427–1441.

### *Poncirus trifoliata* (L.) Raf. (trifoliate orange)

*pBCORc119*—COR11 (S59536) gi 625155—K<sub>S</sub>—106 aa—pI; MW 11.4 kDa (Cai et al., 1995).

*pBCORc115*—COR19 (S59536) gi 625153—K<sub>3</sub>S—179 aa—pI 6.9; MW 19.8 kDa (Cai et al., 1995).

## Reference

Cai, Q., G.A. Moore, and C.L. Guy. 1995. An unusual group 2 LEA gene family in citrus responsive to low temperature. *Plant Mol. Biol.* 29:11–23.

### *Populus alba* L. (white poplar)

(EF639405.1) (ABS12345.1) gi 151515285—228 aa—pI 5.14; MW 25.92 kDa (Kim et al., 2007 unpublished).

## Reference

Kim, Y.-Y., K.-H. Kim, and J.-J. Ku. 2007. Unpublished.

### *Populus alba* × *Populus tremula* L. var. *glandulosa*

*PoDHN* (DQ856592.1) (ABH11546.1) gi 111610121—SK<sub>n</sub>—227 aa—pI 5.13; MW 25.67 kDa (Bae et al., 2006 unpublished).

## Reference

Bae, E.-K., H. Lee, J.-S. Lee, E.-W. Noh, and H.-N. Shin. 2006. Unpublished.

### *Populus* × *canadensis* Moench.

*Dhn1* (EF639408.1) (ABS12348.1) gi 151515291—SK<sub>n</sub>—225 aa—pI 5.26; MW 25.55 kDa (Kim et al., 2007 unpublished).

*Dhn2* (AJ300525.4) (CAC18724.4) gi 29120045—K<sub>n</sub>—616 aa—pI 6.12; MW 68.866 kDa (Caruso et al., 2002).

### *Populus euramericana* (Dode) Guinier ex Piccarolo

*PeuDhn1*—SK<sub>2</sub>—225 aa—gi 29120045—K<sub>3</sub> (Caruso et al., 2002).

***Populus maximowiczii* A. Henry (Japanese poplar)**

(EF639406.1) (ABS12346.1) gi 151515287—SKn—225 aa—pI 5.17; MW 25.72 kDa (Kim et al., 2007 unpublished).

(EF639407.1) (ABS12347.1) gi 151515289—SKn—225 aa—pI 5.17; MW 25.55 kDa (Kim et al., 2007 unpublished).

***Populus tremula* L. var. *daurica***

(EF639391.1) (ABS12331.1) gi 151515257—SKn—227 aa—pI 5.10; MW 25.815 kDa (Kim et al., 2007 unpublished).

***Populus tremula* L. var. *glandulosa***

(EF639404.1) (ABS12344) gi 151515283—SKn—226 aa—pI 5.06; MW 25.66 kDa (Kim et al., 2007 unpublished).

***Populus trichocarpa* Torr. & Gray (black cottonwood) (syn. *Populus balsamifera* ssp. *trichocarpa*)**

(EEE79938.1) gi 222842391—391—YnKn—444 aa—pI 8.98; MW 50.8 kDa (Tuskan et al., 2006).

(XP\_002300665) (XM\_002300629.1) gi 224061921—444 aa—pI 8.98; MW 50.8 kDa (Tuskan et al., 2006).

**Reference**

Tuskan, G.A., S. Difazio, S. Jansson, J. Bohlmann, I. Grigoriev, U. Hellsten, N. Putnam, S. Ralph et al. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313:1596–1604.

***Prunus dulcis* (Mill.) D.A. Webb (almond)**

(DQ061115.1) (AAY98416.1) gi 68510095—YK<sub>4</sub>—272 aa—pI 5.95; MW 28.59 kDa—Abolhassani and Roux unpublished.

**Reference**

Abolhassani and Roux. Unpublished.

*PaRab21* (AF172263.1) (AAD50291.1) gi 5738195—Y<sub>2</sub>SK<sub>2</sub>—202 aa—pI 8.56; MW 21.43 kDa (Campalans et al., 2000).

**Reference**

Campalans, A., M. Pâges, and R. Messegueur. 2000. Protein analysis during almond embryo development. Identification and characterization of a late embryogenesis abundant protein. *Plant Physiol. Biochem.* 38: 449–457.

***Prunus persica* (L.) Batsch. (peach)**

*PpDhn1* (U62486.1) (AAC49658.1) gi 1470109—Y<sub>2</sub>K<sub>9</sub>—468 aa—pI 6.39; MW 49.46 kDa (Artlip et al., 1997).

*PpDhn2* (AY465376.1) (AAS46614.1) gi 43429923—YSK<sub>2</sub>—202 aa—pI 7.95; MW 21.45 kDa (Wisniewski et al., 2006).

*PpDhn3*—(DQ111949.1) (AAZ83586) gi 73762178—SK<sub>2</sub>—249 aa—pI 5.37; MW 28.31 kDa (Bassett et al., 2009).

**References**

Artlip, T.S., A.M. Callahan, C.L. Bassett, and M.E. Wisniewski. 1997. Seasonal expression of a dehydrin gene in sibling deciduous and evergreen genotypes of peach (*Prunus persica* [L.]Batsch.). *Plant. Mol. Biol.* 33:61–70.

Wisniewski, M.E., C.L. Bassett, J. Renaut, R.E. Jr. Farrell, T. Tworkoski, and T.S. Artlip. 2006. Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit. *Tree Physiol.* 26:575–584.

***Pseudotsuga menziesii* (Mirbel) Franco (Douglas fir)**

(221386A) gi 1589647—Kn—164 aa—pI 7.86; MW 17 kDa (Jarvis et al., 1996).

(221386B) gi 1589648—Kn—164 aa—pI 9.02; MW 17 kDa (Jarvis et al., 1996).

(221386C) gi 1589649—Kn—153 aa—pI 8.75; MW 15.9 kDa (Jarvis et al., 1996).

(221386D) gi 158650—Kn—153 aa—pI 8.75; MW 15.921 kDa (Jarvis et al., 1996).

(221386E) gi 158651—Kn—82 aa—pI 9.82; MW 8.88 kDa (Jarvis et al., 1996).

***Pyrus pyrifolia* (Burm. f.) Nakai (Japanese pear)**

(FJ468294.1) (ACK37880) gi 217316936—184 aa—pI 5.4; MW 19.46 kDa (Li et al., 2008 unpublished).

## Reference

Li, X.G., Y. Cong, J. Lin, H. Li, Q.S. Yang, B.L. Sheng, and Y.H. Chang. 2008. Unpublished.

### *Pyrus serotina* Rehder

tr Q9LUX4—K

*Quercus petraea* (Matusschka) Liebl (sessile oak) (Vornam et al., unpublished).

## Reference

Vornam B., Gailing O., and Finkeldey R. Unpublished.

(AM 711635.1) (CAM98306) gi 146424454—112 aa—pI 9.59; MW 12.27 kDa (Vornam et al., unpublished).

(AM711636.1) (CAM98307) gi 146424456—100 aa—pI 9.7; MW 11 kDa (Vornam et al., unpublished).

### *Quercus robur* L. (European oak)

*QrDhn1* (AY607705.1) (AAU06814) gi 51572266—YnSKn—180 aa—pI 5.65; MW 19.58 kDa (Šunderlíková et al., 2009).

*QrDhn2* (AY607706.1) (AAU06815) gi 51572268—Kn—169 aa—pI 7.83; MW 19.01 kDa (Šunderlíková et al., 2009).

*QrDhn3* (AY607707.1) (AAU06816.1) gi 51572270—Kn—112 aa—pI 9.46; MW 12.25 kDa (Šunderlíková et al., 2009).

## Reference

Šunderlíková, V., J. Salaj, D. Kopecky, T. Salaj, E. Wilhem, and I. Matušková. 2009. Dehydrin genes and their expression in recalcitrant oak (*Quercus robur*) embryos. *Plant Cell Rep.* 28:1011–1021.

### *Raphanus sativus* L. (radish)

*RsLEA2* (P21298.1) gi 118575—YSK—184 aa—pI 6.45; MW 19.1 kDa—Raynal et al. (1990)

## Reference

Raynal, M., P. Gaubier, F. Grellet, and M. Delseny. 1990. Nucleotide sequence of a radish cDNA clone coding for a late embryogenesis abundant (LEA) protein. *Nucleic Acids Res.* 18:6132.

### *Retama raetam* (Forsskal) Webb et Berthel. (white weeping broom)

*DHN2* (AF439276.1) (AAL32035.1) gi 16930751—K<sub>2</sub>—66 aa—pI 6.31; MW 7.35 kDa—Pnuli and Mittler 2008. Unpublished.

## Reference

Pnuli, L. and R. Mittler. 2008. Unpublished.

### *Rhododendron catawbiense* Michx. (Catawba rhododendron)

*RcDhn5* (EU549866.1) (ACB41781.1) gi 171188417—SK<sub>2</sub>—240 aa—pI 5.22; MW 27.07 kDa (Peng et al., 2008).

### *Ricinus communis* L. (castor bean)

*Xero* (EQ973879.1) (EEF40786.1) gi 223539193—YSKn—149 aa—pI 7.25; MW 16.72 kDa (Chan et al., 2009 unpublished).

*Xero* (EQ973879.1) (EEF40787.1) gi 223539194—YSKn—146 aa—pI 8.74; MW 16 kDa (Chan et al., 2009 unpublished).

## Reference

Chan, A., D. Puiiu, A. Melake, J. Orvis, Q. Zhao, J. Wortman, T. Utterback, M.J. Rosovitz, J.M. Inman, P. Amedeo, S. Schobel, K. Galinsky, C. Fraser, J. Ravel, and P. Rabinowitz. 2009. Unpublished.

### *Salvia miltiorrhiza* Bunge (sage)

gi 52148123 (AY737725.1) (AAU29458.1)—SK<sub>2</sub>—248 aa—pI 5.19; MW 27.75 kDa—gi 51512229—SK<sub>2</sub> (Liu et al. unpublished).

## Reference

Liu, S.-H., Y.-P. Yan, and Z.-Z. Wang. Unpublished.

### *Solanum commersonii* Dun. ex Poir. (Commerson's wild potato)

*ScDhn1* (Y15813.1) (CAA75798.1) gi 2689251 YSK<sub>2</sub>—157 aa—pI 8.01; MW 16.658 kDa (Baudo et al., 1996).

*ScDhn2* (AF386075) (AAK66763) gi 14538005—SK—199 aa—pI 6.62; MW 23.53 kDa (Seppanen et al., 2001 unpublished).

## Reference

Baudo, M.M., L.A. Meza-Zepeda, E.T. Palva, and P. Heino. 1996. Induction of homologous low temperature and ABA-responsive genes in frost resistant (*Solanum commersonii*) and frost sensitive (*Solanum tuberosum* cv. Bintje) potato species. *Plant Mol. Biol.* 30:331–336.

### *Solanum soganandinum* Ochoa

*Dhn10* (AF542504) (AAN37899) gi 23506601—KS—86 aa—pI 7.2; MW 10 kDa (Rorat et al., 2004).

*Dhn24* (AY292655) (AAP44575) gi 31335281—SK<sub>3</sub>—210 aa—pI 5.25; MW 23.8 kDa (Rorat et al., 2006).

## Reference

Rorat, T., B.M. Stahala, W.J. Grygorowicz, B. Wojtowicz, Z. Yin, and P. Rey. 2006. Expression of SK<sub>3</sub>—type dehydrin in transporting organs associated with cold acclimation in *Solanum* species. *Planta* 224:205–221.

### *Solanum tuberosum* L. (potato)

*StDhn1* (X83597) (CAA58576)—134 aa—pI 8.14; MW 14.2 kDa (Baudo et al., 1996).

*ci7* (U69633) (AAB53203) gi 2055384—SK<sub>3</sub>—209 aa—pI 5.36; MW 23.7 kDa (Kirch et al., 1997).

## Reference

Kirch, H.-H., J. Van Berkel, H. Glaczinski, F. Salamini, C. and Gebhardt. 1997. Structural organization, expression and promoter activity of a cold-stress-inducible gene of potato (*Solanum tuberosum* L.). *Plant Mol. Biol.* 33:897–909.

### *Sorghum bicolor* L. (sorghum)

*DHN1* (U11696.1) (AAA19693) gi 509549—YSK<sub>2</sub>—154 aa—pI 9.67; MW 16.27 kDa (Wood and Goldsbrough, 1994 unpublished).

*DHN2* (U63831.1) (AAB05927.1) gi 1488312—SKn—106 aa—pI 9.4; MW 10.97 kDa (Whitsitt and Mullet, 1996 unpublished).

## References

Whitsitt, M.S. and J.E. Mullet. 1996. Characterization and mapping of drought responsive genes in *Sorghum*. Unpublished.

Wood, A.J. and P.B. Goldsbrough. 1994. Characterization and expression of dehydrins in drought stressed *Sorghum bicolor*. Unpublished.

### *Spinacia oleracea* L. (spinach)

*Cap85* (M96259.1) (AAB88628.1) gi 2673888—K<sub>11</sub>—535 aa—pI 5.94; MW 61.5 kDa (Neven et al., 1993).

### *Stellaria longipes* (Goldie) Coville (longstalk starwort)

*H26* (Z21500.1) (CAA79709) gi 433652—SK<sub>3</sub>S—173 aa—pI 10.49; MW 19.37 kDa (Zhang et al., 1996).

## Reference

Zhang, X.H., M.M. Moloney, and C.C. Chinnappa. 1996. Analysis of an ABA- and osmotic stress-inducible dehydrin from *Stellaria longipes*. *J. Plant Physiol.* 149:617–622.

### *Tamarix hispida* Willd.

*ThDHN* (FJ627947.1) (ACN42853.1) gi 224384119—204 aa—pI 5.61; MW 22.76 kDa (Wang et al., 2009 unpublished).

## Reference

Wang, Y., R. Zhang, C. Gao, and G. Liu. 2009. Unpublished.

### *Taraxacum officinale* Desf. (common dandelion)

(DQ160121.1) (ABA27060.1) gi 75756011—K—107 aa—pI 6.6; MW 12.345 kDa (Hulzink et al., 2005 unpublished).

## Reference

Hulzink, R.J.M., P.J. van Dijk, and A. Biere. 2005. Isolation and characterization of candidate genes for pathogen and herbivory defense in common dandelion (*Taraxacum officinale*) upon salicylic acid or methyl jasmonate treatment. Unpublished.

### *Tithonia rotundifolia* (Mill.) S.F. Blake (Mexican sunflower)

*Dhn 1* (AJ250127.1) (CAC80717.1) gi 18076154—Y<sub>3</sub>SK<sub>2</sub>—244 aa—pI 7.36; MW 25.02 kDa (Cavallini et al., 2007 unpublished).

## Reference

Cavallini, A., T. Giordani, L. Natali, and A. Pardini. 2007. Unpublished.

### *Triticum aestivum* L. (common wheat)

*Wcs200* (AAB31285)—Kn—pI 6.5; MW (200 kDa) (Quellet et al., 1993).

*Wcs180*—Kn—pI 6.5; MW (180 kDa) (Houde et al., 1995).

*Wcs120* (M93342) (AAA34261)—K<sub>6</sub>—390 aa—pI 7.02; MW 39 kDa (50 kDa) (Houde et al., 1992a).

*Wcs66* (L27516) (AAA21819)—K<sub>7</sub>—469 aa—pI 6.74; MW 46.8 kDa (66 kDa) (Chauvin et al., 1994).

*Wcs40*—Kn—pI 7.3; MW (40 kDa) (Houde et al., 1995).

*Wcs726/Wcor726* (U73213) (AAB18204)—Kn—124 aa—pI 7.04; MW 12.7 kDa (Danyluk and Sarhan, 1996) NCBI.

*Wcs80/Wcor80* (U73212) (AAB18203)—Kn—93 aa—pI 8.05; MW 9.6 kDa (Danyluk and Sarhan 1996) NCBI.

*Cor39* (AF058794) (AAC14297)—K<sub>6</sub>—391 aa—pI 6.92; MW 39 kDa (50 kDa) (Guo et al., 1992).

*Wdhn13* (AB076807) (BAC011112)—K<sub>3</sub>—124 aa—pI 8.01; MW 12.8 kDa (Ohno et al., 2003).

*Wcor410a* (L29152) (AAA20189)—SK<sub>3</sub>—262 aa—pI 5.19; MW 28 kDa (Danyluk et al., 1994, 1998).

*Wcor410b* (U73210) (AAB18201)—SK<sub>3</sub>—268 aa—pI 5.25; MW 28.8 kDa (Danyluk et al., 1994, 1998).

*Wcor410c* (U73211) (AAB18202)—SK<sub>3</sub>—259 aa—pI 5.2; MW 27.9 kDa (Danyluk et al., 1994, 1998).

*Wcor825* (U73215) (AAB18206)—KS—73 aa—pI 8.08; MW 8.1 kDa—Danyluk and Sarhan (1996) unpublished.

*Rab15* (X59133)—YSK<sub>2</sub>

## References

Guo, W., R.W. Ward, and M.F. Thomashow. 1992. Characterization of a cold-regulated wheat gene related to *Arabidopsis Cor47*. *Plant Physiol.* 100:915–922.

Danyluk, J., M. Houde, E. Rassart, and F. Sarhan. 1994. Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant *Gramineae* species. *FEBS Lett.* 344:20–24.

Ohno, R., S. Takumi, and C. Nakamura. 2003. Kinetics of transcript and protein accumulation of a low-molecular-weight wheat LEA D-11 dehydrin in response to low temperature. *J. Plant Physiol.* 160:193–200.

Quellet, F., M. Houde, and F. Sarhan. 1993. Purification, characterization and cDNA cloning of the 200 kDa protein induced by cold acclimation in wheat. *Plant Cell Physiol.* 34:59–65.

### *Triticum turgidum ssp. durum* (Desf.) Husn. (durum wheat)

*Dhn1* (AM180929.1) (CAJ5605.1) gi 121489513—YSKn—149 aa—pI 9.52; MW 15.105 kDa (Rampino et al., 2006).

*Dhn2* (AM180930.1) (CAJ56060.1) gi 121489515—SK<sub>2</sub>—133 aa—pI 9.66; MW 13.26 kDa (Rampino et al., 2006).

*Dhn3* (AM180931.1) (CAJ56061.1) gi 121489517—YSKn—149 aa—pI 8.01; MW 15.244 kDa (Rampino et al., 2006).

*Dhn4* (AM180932.1) (CAJ56062.1) gi 121489519—Kn—93 aa—pI 6.79; MW 9.66 kDa (Rampino et al., 2006).

*Dhn5* (AY619566)—YSK<sub>2</sub>—227 aa (Brini et al., 2007a).

***Trollius ledebourii*** (papilio troilus)

tr Q43668—K<sub>2</sub>.

***Vaccinium corymbosum*** L. (blueberry)

*BbDhn1* (AF030180) (AAB84258) gi 2613040—K<sub>5</sub>—314 aa—pI 6.63; MW 34.3 kDa (Levi et al., 1999).

*BbDhn2* (AF222738.1) (AAF34603.1) gi 6979696—Kn—131 aa—pI 6.53; MW 14.32 kDa (Panta and Rowland, 2000 unpublished).

*BbDhn3* (AF222739.1) (AAF34604.1) gi 6979698—Kn—208 aa—pI 6.67; MW 22.95 kDa (Panta and Rowland, 2000 unpublished).

*BbDhn4* (AF222740.1) (AAF34605.1) gi 6979700—K<sub>3</sub>—218 aa—pI 6.58; MW 23.8 kDa (Panta and Rowland, 2000 unpublished).

*BbDhn5* (AF222741.1) (AAF34606.1) gi 6979702—Kn—320 aa—pI 6.2; MW 35.17 kDa (Panta and Rowland, 2000 unpublished).

*BbDhn6* (AY660959) (AAT76302) gi 50380069—K<sub>2</sub>—101 aa—pI 8.44; MW 10.9 kDa (Dhanaraj et al., 2005).

*BbDhn7/Cor11* (AY660960.1) (AAT76303.1) gi 50380093—K<sub>2</sub>—108 aa—pI 7.97; MW 11.8 kDa (Dhanaraj et al., 2005).

**Reference**

Panta G.R. and L.J. Rowland. 2000. Cloning and characterizing of blueberry (*Vaccinium corymbosum* section *Cyanococcus*) dehydrins. Unpublished.

***Vaccinium vitis-idaea*** L. (cranberry)

*Cor11* (FJ429387.1) (ACJ54952.1) gi 213959408—Kn—108 aa—pI 8.58; MW 11.638 kDa (Wang and Zhang, 2008 unpublished).

**Reference**

Wang, Q.J. and Z. Zhang. 2008. Unpublished.

***Vicia monantha*** Retz. (barn vetch)

*DHNa* (AB506694.1) (BAH70483.1) gi 239735425—YKn—195 aa—pI 5.97; MW 20.21 kDa (Nada et al., 2009 unpublished).

*DHNb* (AB506695.1) (BAH70484.1) gi 239735427—YKn—138 aa—pI 5.92; MW 14.17 kDa (Nada et al., 2009 unpublished).

**Reference**

Nada, A.M., H.M. Abd el-Halim, and A.H. Fahhad. 2009. Cloning of two dehydrin genes from the halophytes of the Egyptian north west coastal region. Unpublished.

***Vigna unguiculata*** (L.)Walp. (cowpea)

*Dhn1* (AF159804) (AAF07274) gi 6358640—Y<sub>2</sub>K—259 aa—pI 5.97; MW 26.5 kDa (Ismail et al., 1999a).

***Vitis riparia*** Michx. (frost grape)

*Dhn1a*—(AY706987.1) (AAW58105.1) gi 57903608—YSK<sub>2</sub>—130 aa—pI 9.44; MW 13.9 kDa (Xiao and Nassuth, 2006).

***Vitis vinifera*** L. (grapevine)

*Dhn1a* (AY706989.1) (AAW58106.1) gi 57903611—YSK<sub>2</sub>—130 aa—pI 9.27; MW 13.92 kDa (Xiao and Nassuth, 2006).

*Dhn1b* (AY706990.1) (AAW58107.1) gi 57903613—YSK<sub>2</sub>—124 aa—pI 9.04; MW 13.23 kDa (Xiao and Nassuth, 2006).

***Xerophyta viscosa*** Baker (resurrecting hope)

gi 30349507 (AY266308.1) (AAP22171)—YSK—101 aa—pI 8.02; MW 11.16 kDa (Baker et al., 2003 unpublished).

## Reference

Baker, B., S.G. Mundree, and J.A. Thomson. 2003.

### *Zea mays* L. (maize)

*DHN1* (P12950) gi 118484—YSK<sub>2</sub>—168 aa—pI 9.1; MW 17.16 kDa (Close et al., 1989).

*DHN2* (FJ436407.1) (ACJ65012.1) gi 215274574—SKn—281 aa—pI 5.57; MW 30.653 kDa (Xing, 2008 unpublished).

*RAB17* (X15994)—YSK<sub>2</sub> (Vilardell et al., 1990).

*COR410* (EU966442.1) (ACG38560.1) gi 195638184—SKn—291 aa—pI 6.05; MW 31.497 kDa (Alexandrov et al., 2009).

*COR410* (NP\_001147478) (NM\_001154006.1)—SKn—291 aa—pI 6.18; MW 31.497 kDa (Alexandrov et al., 2009).

*Dhn13* (*dehydrin 13*) (NP\_001151632.1) (NM\_001158160.1) gi 226501978—KS—102 aa—pI 6.59; MW 11.555 kDa (Alexandrov et al., 2009).

*Dhn13* (NP\_001150115.1) (NM\_001156643.1) gi 226494546—KS—107 aa—pI 6.34; MW 12.098 kDa (Alexandrov et al., 2009).

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# 11 Behavior of Water in Plants at Low and Ultralow Temperatures

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11.1 FREEZING PROCESS AT LOW TEMPERATURES

It is possible to measure changes in the properties of water in living systems that correlate with physiological functions. Changes in dynamic properties of water at different hydration levels indicate the existence of different fractions of water, which may vary in structure and property and presumably play different biological roles (Sun, 2002). Studies have identified the existence of at least four or five fractions of water, presumably relating to different interactions between water and cellular constituents (Pissis et al., 1996; Ratkovic, 1987; Sun, 2000; Vertucci, 1990).

One species of larch (*Laryx dahurica*), for example, survives in the most northerly forests of Siberia, where temperatures commonly reach  $-65^{\circ}\text{C}$  to  $-70^{\circ}\text{C}$ . Nonacclimated seedlings of winter rye (*Secale cereale*), for example do not survive temperatures lower than  $-4^{\circ}\text{C}$  or  $-5^{\circ}\text{C}$ . However, when acclimated, seedlings survive temperatures as low as  $-28^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$ . Both of these examples from the plant kingdom have different mechanisms that allow them to survive such low temperatures (Figure 11.1).

How do plants freeze? Apart of the problem in trying to solve how plants freeze has been the difficulty in determining where freezing in a plant is initiated and how the freezing proceeds propagation throughout the plant. Several questions still remain unanswered: Can plants supercool without extrinsic nucleators? How many nucleation events are needed for a whole plant to freeze? Do barriers exist within plants that influence the rates or ways of ice propagation? How do cold acclimation, antifreeze proteins, anti-nucleators, and elevated  $\text{CO}_2$  affect ice nucleation? And what determines the natural patterns of frost injury present in a field after a freezing event (Wisniewski et al., 2009)? Plants can use both tolerance and avoidance strategies for surviving frost (Sakai and Larcher, 1987; Wisniewski et al., 2009).

The growth of extracellular ice is the most common freezing pattern in plants, which, for example, occurs in the leaves of most plants and in the bark of woody species. Most of this ice grows at the expense of water drawn from within the non-frozen cells. By freezing extracellularly, the cells avoid freezing, but at the expense of suffering partial or extensive dehydration. Plants are not able to tolerate severe extracellular dehydration. Dehydration to the certain level can cause lethal stress (Table 11.1).

The temperature at which ice melts ( $T_m$ ) is well determinable in contrary to the freezing temperature ( $T_f$ ). It is a common knowledge that  $T_m$  and  $T_f$  of pure water is  $0^{\circ}\text{C}$ . Pure water has the ability

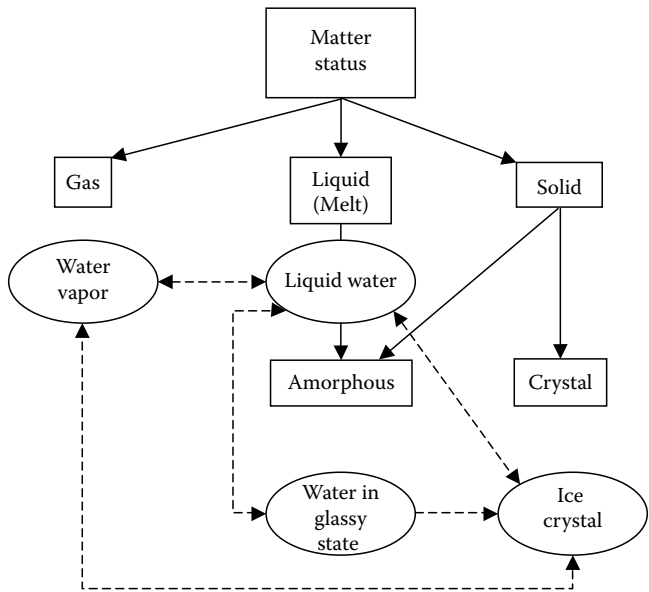


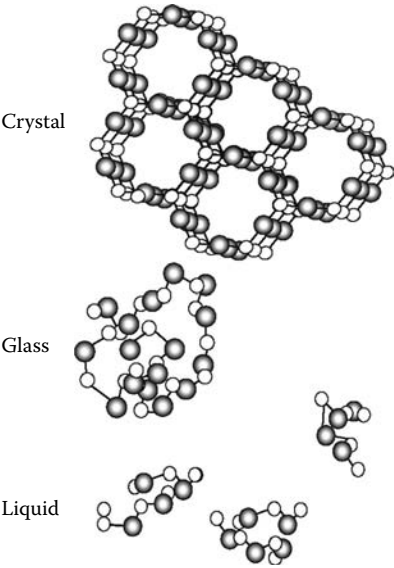
FIGURE 11.1 Status of matter with examples for water.

**TABLE 11.1**  
**Temperature Boundary Marks with Water Physiological Stress Meaning**

| Temperature [°C] | Water/Ice/Glass Transition   | Examples in Plants                              |
|------------------|------------------------------|-------------------------------------------------|
| 0                | Pure water freezing          | Rain drops, dew, on the surface of plants       |
| 0~–38            | Supercooling                 | Trees buds                                      |
| 0~–38            | Heterogeneous ice nucleation | Plant-intrinsic ice nuclei                      |
| ~–38             | Homogeneous ice nucleation   | Limit for supercooling plants                   |
| ~–80             | Ice forms transfer           | Lowest temperature on the Earth                 |
| –135             | Water glass transition       | Cryopreservation in liquid nitrogen vapor phase |
| –196             | Liquid nitrogen              | Cryopreservation in liquid nitrogen             |

to supercool to temperatures as low as  $-40^{\circ}\text{C}$  (Rajashekar and Burke, 1996). Pure water freezes at much warmer, subzero temperatures due to the presence of heterogeneous nucleators that induce ice crystal formation (Franks, 1985) (Figure 11.2).

Freezing is precisely defined as a *first-order phase transition* which involves the crystallization of liquid water. First-order transitions have a latent heat of the transition, they occur at a well-defined transition temperature, and the heat capacity shows discontinuity at the transition temperature. If this water crystallizes from an aqueous solution, then the process of freezing is accompanied by a gradual increase in the concentration of all soluble components in the residual liquid phase. The freeze concentration of an intracellular solution can have dangerous influence on the viability of cells. When the extracellular freezing is involved, the injury is due to the concentration effect. When a plant at the low temperature is accompanied by freezing, damage arises mainly from factors such as changes in ionic activities, reduction in diffusion rates, and a disruption of the energetic balance which is responsible for the maintenance of biologically significant structures—membranes, enzymes, and other macromolecular complexes—and differential changes in the rates of coupled reactions.



**FIGURE 11.2** Water molecules' arrangement in the three states; water molecules in ice crystal are ordered in a regular lattice; water molecules in glass are disordered but are rigidly bound; water molecules in liquid are disordered and are not rigidly bound but they form clusters of molecules.

The melting of a solution is determined by the concentration and types of substances dissolved in the solution. A simple equation describes the relationship between the osmotic potential ( $\Psi_s$  [osmol]) of a solution in equilibrium with ice at subzero temperature ( $T$  [°C]).

$$\Psi_s = \frac{T}{1.86} \quad (11.1)$$

*Freezing point depression* describes the phenomenon that the freezing point of a liquid (a solvent) is depressed when another compound is added. A solution has a lower freezing point than that of a pure solvent. In plants the solutes are concentrated by removing the excess of pure solvent, water. At the freezing point (Beurroies et al., 2004), the solid phase and the liquid phase have the same chemical potential, meaning that they are energetically equivalent. Often during freezing, a solute only dissolves in the liquid water and not in the ice. This means that when such a solute is added, the chemical potential in the liquid phase is decreased by water dilution, but the chemical potential of the water in the solid phase, ice, is not affected.

The chemical potential of a solution is temperature dependent. At some temperatures, either in the solid or in the liquid phase, it has a lower chemical potential and is more energetically favorable than the other phase. This means in turn that the equilibrium between the ice and liquid phase is established at lower temperature for a solution than for a pure liquid; the freezing point is depressed.

The freezing/melting point depression,  $\Delta T_m$ , is defined as

$$\Delta T_m = T_{m(\text{water})} - T_{m(\text{solution})} \quad (11.2)$$

The difference between the freezing points of the pure solvent and the solution is expressed by following equation

$$\Delta T_m = K_m m_B \quad (11.3)$$

where  $K_m$  is cryoscopic constant, which is dependent on the properties of the solvent,  $m_B$  is the molality of the solution, calculated by taking dissociation into account since the freezing point depression is a colligative property, dependent on the number of particles in the solution. This is most easily done by using the van't Hoff factor  $i$  as  $m_B = m_{\text{solute}} i$ . The factor  $i$  accounts for the number of individual particles formed in solution, where  $i$  is, for example, equal to 1 for sugar in water and 2 for sodium chloride in water. So the cryoscopic constant can be calculated as follows (Workmaster et al., 1999)

$$K_m = \frac{R \Delta T_m^2 M}{\Delta H_m} \quad (11.4)$$

where

$R$  is the gas constant

$\Delta T_m$  is the freezing/melting point of the pure solvent (in Kelvin)

$M$  is the molar mass of the solvent

$\Delta H_m$  is the enthalpy of fusion per mole of the solvent

This formula is valid for diluted solution and for ideal solutions.

At high concentrations, Equation 11.2 is less precise due to the approximations used in its derivation and in any non-ideality of the solution. If solute is soluble in solid solvent, one of the key

assumptions used in deriving the formula is not true. In this case the effect of the solute on the freezing point must be determined from the phase diagram of the mixture.

The freezing point of pure water is 0°C. The solutes in plant cells depress the freezing point of water below 0°C. The extent to which the freezing point is depressed below 0°C is proportional to the concentration of dissolved solute particles (see Equations 11.2 and 11.3); a 1 molal solution of an ideal non-ionized solute freezes at -1.86°C and has an osmotic potential of -2.27 MPa. Based on this relationship, the freezing point depression of any unknown solution can be calculated from the osmotic potential, as comes from Equation 11.1

$$\Delta T_m = \frac{\Psi_s}{1.221} \quad (11.5)$$

where

$\Psi_s$  is osmotic potential of solution (MPa)

1.221 is constant (MPa deg<sup>-1</sup>)

Since this equation is valid for solutions at 0°C (273 K), we must correct the equation to room temperature by multiplying the equation by the ratio of the absolute temperatures (room temperature in K/273 K)

$$\Delta T_m = \frac{\Psi_s}{1.221 T_a / 273} \quad (11.6)$$

where  $T_a$  is room temperature in K. Freezing point depression is generally produced by concentration of solutes in tissue.

With freezing, the extracellular solution becomes more concentrated and water therefore leaves the cell until the gradient in chemical potential is equilibrated, establishing osmotic equilibrium across the plasma membrane. This new equilibrium results from the concentration of the intracellular solutes. The equilibrium cell volume  $V$  is a function of the extracellular solute concentration and has traditionally been expressed by the Boyle–van't Hoff equation

$$V = \Psi_s^o (V^o - b) \left( \frac{1}{\Psi_s} \right) + b \quad (11.7)$$

where

$\Psi_s$  is the osmolality of the extracellular solution

$b$  is the osmotically inactive volume of the cell

$V^o$  and  $\Psi_s^o$  are the cell volume and the extracellular osmolality, respectively, for the isotonic condition

Note that the Boyle–van't Hoff equation is derived by assuming that the cell contents form a thermodynamically ideal, dilute solution. The Boyle–van't Hoff plot can show the relative cell volume as a function of inverse osmolality. The extrapolation of a straight line to infinite osmolality has been used to estimate the osmotically inactive fraction of the cell volume (Burke et al., 1976; Gusta et al., 1975). The rate of movement of water across a membrane is limited by the permeability properties of the membrane. The rate at which the cell loses water or input water after thawing is a function of the magnitude of the water vapor gradient and the

permeability of the plasma membrane (the area of the membrane and its conductivity to water) and is given by the following relation

$$\frac{dV_w}{dt} = L_p A R T (\Psi_i - \Psi_e) \quad (11.8)$$

where

- $V_w$  is the water volume of the cell
- $t$  is time
- $L_p$  is the hydraulic conductivity
- $A$  is the membrane surface area
- $R$  is the gas constant
- $T$  is the absolute temperature
- $\Psi_i$  is the intracellular water potential
- $\Psi_e$  is the extracellular water potential

For vapor phase see Equation 11.12.

In practical sense the osmotic potential of cell samples can appear after the tissue freezes to the temperature at which the cells ruptured. The osmotic potential after freezing and thawing cannot reflect the actual situation during freezing. The intact tissue volume can mix with the apoplastic water volume and, after thawing, the osmotic potential can be much higher because of the dilution effect of extracellular water with intracellular sap.

The effect of osmotic potential on the freezing point depression also holds for non-ideal solutions. However, the freezing point depression is non-linear with concentration changes during dehydration. Water potential (MPa at 0°C) can be derived by the following empirical equation (Crafts et al., 1949; Wisniewski et al., 2009):

$$\Psi_s = -1.206 \Delta T_m + 0.0021 \Delta T_m^2 \quad (11.9)$$

During lowering the temperature, the sample is usually supercooled a few degrees below its freezing point. After the induction of crystallization, as the heat of fusion is released, the sample temperature rises to its freezing point, at its temperature equilibrium. Similarly, the temperature at which ice crystals start to melt was taken as the equilibrium thawing temperature.

### 11.1.1 SUPERCOOLING

Supercooling occurs when solution freezes at temperature lower than at the freezing point. Supercooling is important to the surviving plants' low temperature stress. The ice nuclei have cluster form as like ice lattice in shape and size critical for certain temperature (Šesták and Zámečník, 2007). During heterogenic ice nucleation, the critical size and shape are formed from external impurity with the ability to start nucleation. In the nature the most common external ice nucleators come from frozen dews, snowflakes, small ice crystals, or epidemic ice-nucleating bacteria on plants. When water is cooled below 0°C, and in the absence of ice nuclei it can be supercooled down to approximately -40°C. As an example, pure water droplets of about 10 μm in diameter supercooled to -38.1°C, provided no heterogeneous nucleators were presented by Rasmussen and Mackenzie (1972). The spontaneous nucleation temperature is influenced by the concentration of solutes reflecting in freezing point depression, as shown in the following equation:

$$T_h = -(38.1 + 1.86 \Delta T_m) \quad (11.10)$$

where

- $\Delta T_m$  is the freezing point depression for the solution (in K)
- $T_h$  is the homogeneous nucleation temperature (in °C)

The proportionality constant has a theoretical value 1.86; for most low molecular substances the value is 2 and for few polymers this constant lies between 4 and 6 (Franks, 1985).

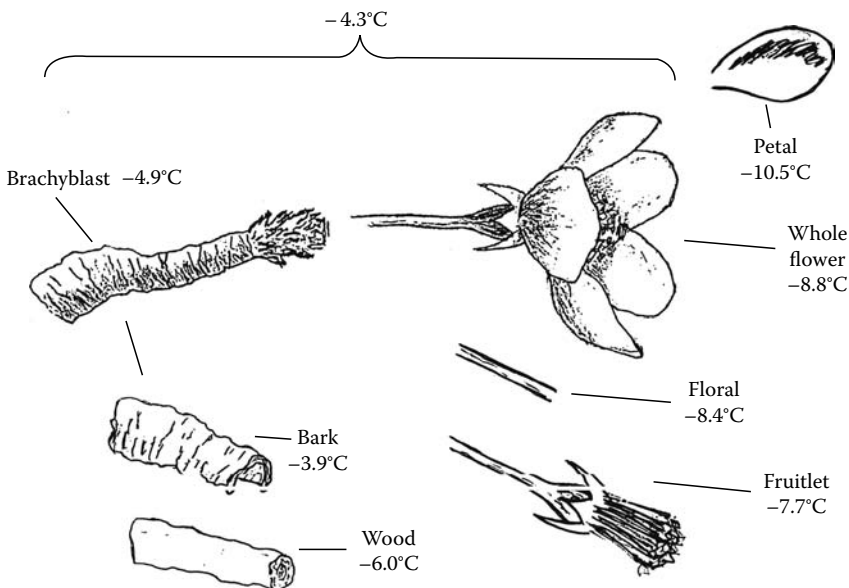
So solution in plant cells may be supercooled to a lower temperature than supercooled pure water, usually from  $-41^{\circ}\text{C}$  to  $-47^{\circ}\text{C}$ , because cell solutes depress the homogenous nucleation temperature. In small holes in plants the water can be supercooled to below  $-40^{\circ}\text{C}$ . In laboratory experiments with the distilled water, supercooling cannot reach temperature below  $-20^{\circ}\text{C}$ . The supercooling of the cell contents would avoid ice crystal formation. However, if the supercooling limit of cells is exceeded, then intracellular freezing occurs more often, and it is generally accepted that intracellular ice formation is lethal for plants (Guy, 1990). Most tropical plants are unable to protect themselves against freezing, but most species of temperate plants can acclimate to cold, so the ice forms only outside the cells as the temperature drops.

Deep supercooling, from about  $-15^{\circ}\text{C}$  down to a limit of about  $-40^{\circ}\text{C}$ , occurs in many temperate woody plants such as apple and maple tree. Deep supercooling is the only way for woody plants to withstand severe winters. Deep supercooling is limited to only certain organs or tissues. For example, in apple tree the cells of the xylem parenchymal cells deep supercool. The highest ice nucleation activity (INA) was found in apple twig bark  $-3.9^{\circ}\text{C}$  and brachyblast  $-4.9^{\circ}\text{C}$ . The average INA was not exceeded in xylem sap  $-7.4^{\circ}\text{C}$  and in extract from stem  $-6.7^{\circ}\text{C}$  (Bilavcik et al., 1997) (Figure 11.3).

Maple, deep supercool but the bark freezes extracellularly (Ashworth et al., 1988). In forsythia and maple, cells in flower bud organs supercool but the bud scales and nearby stems, again, freeze extracellularly (Ashworth, 1990; Ishikawa et al., 1997).

More unusual phenomena may occur in the extreme freezing tolerance; below the  $-40^{\circ}\text{C}$  limit of water drops to deep supercool. In the xylem parenchyma of the red osier dogwood, ice forms between the cell wall and the plasma membrane outside the protoplast, without damaging it (Ashworth, 1996).

The deep supercooling of the xylem is found in many temperate deciduous trees. The xylem ray parenchyma cells are generally believed to deep supercool and as such remain viable, whereas the freezing of these cells is lethal. However, with the nuclear magnetic resonance (NMR) images resolution investigation of full moon maple, it is not possible to confirm whether only the xylem ray



**FIGURE 11.3** An average ice nucleation activity (potential supercooling temperature) of an apple blooming twig parts. Twig parts were detached and ice nucleation were measured by thermobattery with thermocouple in each drop in connection as differential thermal analysis. (From Bilavčik et al., 1997.)

parenchyma supercool as the images appear as the entire xylem remained unfrozen. In contrast, in full moon maple flower buds, deep supercooling was detected between the mature pith and not in the xylem (Ishikawa et al., 2009).

### 11.1.2 ICE NUCLEATION

The critical size for a homogenous nucleus to begin ice crystal growth is 45,000 water molecules at  $-5^{\circ}\text{C}$ , 650 water molecules at  $-20^{\circ}\text{C}$ , and only 70 water molecules at  $-40^{\circ}\text{C}$  (Vali, 1995). Below the critical size a spontaneous dissolution of the ice nuclei occur due to solubility. Thus, the temperature of homogenous nucleation is a function of sample volume and of time. But the function is an exponential one, with nucleation decreasing so rapidly above  $-40^{\circ}\text{C}$  that it is rarely seen more than a few degrees above  $-40^{\circ}\text{C}$  (Zachariassen and Kristiansen, 2000). The probability of a volume  $V$  of pure water freezing in time  $\Delta t$  due to homologous nucleation is  $J(T)$  times  $V$  times  $\Delta t$ .  $J(T)$  is nucleation rate at temperature  $T$ , determined by the empirically derived equation

$$J(T) = 6.8 \times 10^{-50} e^{-3.9T} \quad (11.11)$$

for  $J(T)$  in  $\text{m}^3 \text{s}^{-1}$  and  $T$  in  $^{\circ}\text{C}$  (Vali, 1995). The  $e^{-3.9T}$  factor means that the probability of nucleation increases by about 50 for each drop of  $1^{\circ}\text{C}$  or by over 6 million for a  $4^{\circ}\text{C}$  temperature drop (Ashworth and Wisniewski, 1991). Although volume and time are linear components in the probability, the exponential temperature component means that the probability rapidly goes from zero to one in the temperature range between  $-38^{\circ}\text{C}$  and  $-42^{\circ}\text{C}$  (being very close to  $-40^{\circ}\text{C}$  for all practical purposes).

The initial formation of extracellular ice in plants is under the control of ice nucleators (Brush et al., 1994) and antifreeze proteins (Hon et al., 1994). Extracellular ice is relatively benign, but acts as a nucleation site for water vapor drawn out of the cell, thereby desiccating the cell. As a consequence, cell volume decreases, but the cell wall remains appressed to the plasma membrane, and ice crystals generally forms between cell wall junctions. It confirms that in this stable state that most cold-stressed tissues overwinter at subzero temperatures. A hydrophilic protein with the potential of conferring desiccation tolerance was found in the vascular transition zone of the wheat crown, which is critical tissue for winter survival (Houde et al., 1995). However, ice-driven desiccation can continue to the point of nonreversible cellular damage. The formation of ice within plants and mechanisms of freezing tolerance have been discussed in review (Gusta et al., 2009a).

The INA of bacteria has been well characterized (Lindow, 1995) and the protein, and corresponding gene, have been isolated and identified (Lindow et al., 1989). In contrast, while INA of plant origin has been commonly observed, especially in woody plants (Ashworth and Kieft, 1995; Lindow, 1995), plant ice nucleation active compounds have not been identified and remain ambiguous, as does an understanding of their origin, development, distribution, turnover, and the role in adaptation to freezing temperatures. The composition of plant ice nucleation active substances is referred in wide scale, from a soluble (Embuscado et al., 1996; Krog et al., 1979) or structural (Chalkerscott, 1992) polysaccharide (Gross et al., 1988) to a protein (Constantinidou and Menkissoglu, 1992) or to a complex molecule such as a phospholipid. The temperature at which extrinsic nucleation occurs in plants appears to be an adaptive process requiring *de novo* synthesis, as it is influenced by cold acclimation (Ball et al., 2002; Beerling et al., 2001; Ishikawa et al., 2009; Lutze et al., 1998).

Although ice nucleation is frequent in woody plants, it also occurs in some herbaceous plants such as *Veronica* (Kaku, 1973) and is often a constitutive feature, present in plants during the whole year. The intrinsic nucleators extracted from plants result in a loss of ice nucleation activity, indicating that a structural component may be essential for them to improve their ice nucleation ability (Griffith and Antikainen, 1996). As a rule, inoculation with ice crystals is a prerequisite for the initiation of extracellular freezing of a cell that is not already in a considerable state of supercooling.

Subzero temperatures experienced by plants in field conditions do not necessarily accompany the occurrence of ice nucleation. Even in the absence of extrinsic ice nucleators, the tissues of cold-hardy plants that undergo extracellular freezing readily freeze at high subzero temperatures and avoid excessive supercooling of the tissues. *Lobelia* species, for example, appear to positively control the initiation of freezing by producing ice-nucleating substances. By the freezing of water involving latent heat of freezing, it prevents too great a drop in temperature in the hollow inflorescence axes of *Lobelia telekii* (Krog et al., 1979).

Why the scale tissues of Japanese azalea freeze spontaneously during cooling to  $-7^{\circ}\text{C}$  was determined. The outer and inner bud scales ( $-5.9^{\circ}\text{C}$  and  $-6.8^{\circ}\text{C}$ , respectively) which freeze first and act as ice sources had the highest ice nucleating activity, while the florets which remain supercooled lacked effective INA ( $-13^{\circ}\text{C}$  to  $-16^{\circ}\text{C}$ ). In the twigs, the bark tissues that undergo extracellular freezing had a high INA of  $-6.3^{\circ}\text{C}$ . The same findings with higher nucleation activity in bark of apple twigs at  $-3.9^{\circ}\text{C}$  and at  $-4.9^{\circ}\text{C}$  in brachyblast were observed (Bilavcik and Zámečník, 1996).

In contrary xylem and pith tissues of Japanese azalea which undergo deep supercooling had low INA ( $-12.8^{\circ}\text{C}$  and  $-12.4^{\circ}\text{C}$ , respectively) (Ishikawa et al., 2009). The INA of each tissue closely corresponded with its freezing behavior.

These INA values in scale tissues of Japanese azalea were affected only slightly by a fourfold increase in the tissue amount and also by an increase in the incubation time at subzero temperatures. The high INA in the outer scales and bark tissues was unaffected by the homogenization of the tissues into fine powders and subsequent extensive washings (Ishikawa et al., 2009). The substances responsible for the INA in each tissue have different sensitivities to heat treatment: the high INA in the bud scale tissues was unaffected by autoclaving ( $121^{\circ}\text{C}$  for 15 min), while the high INA in the bark tissues was sensitive to autoclaving (decreased from  $-11^{\circ}\text{C}$  to  $-12^{\circ}\text{C}$ ). The subcellular fractionation of the ice nucleation activities in the bark by differential centrifugation revealed that the ice nucleation activities were associated with the cell-wall-rich fraction (Ishikawa et al., 2009). These results imply that these ice nucleation activities do not arise from macrostructures such as the presence of trichomes or the outer scales or bark situated in the outermost part of the buds or stems, but from substances tightly bound to the cell walls (Ishikawa et al., 2009; Muryoi et al., 2004). This suggests that these ice nucleation activities are specific to the tissues and their age (Luis et al., 2007).

Nevertheless, several intrinsic nucleators have been partly characterized. Mucilage and carbohydrates, respectively, appear to be the nucleators in *Opuntia* and in giant high-altitude African species of *Lobelia* (Goldstein and Nobel, 1991). Interestingly, cell wall arabinoxylans may have an opposite antifreeze effect in rye and barley crowns and seeds (Kindel et al., 1989; Olien and Smith, 1977). The nucleator in peach and apple trees nucleator appears neither proteinaceous nor lipidic in composition (Ashworth et al., 1985). In contrast, nucleators from rye are proteinaceous, though they also included phospholipids and carbohydrate components (Vali, 1995). Thus, all plant-intrinsic ice nucleators are not of one kind.

### 11.1.3 SEASONAL INA DEPENDING

The flower bud scales of Japanese azalea showed seasonal changes in INA. The outer and inner scales of late August flower buds (just completed morphological development) had low INA; from there on INA increased and attained the maximum activity in late October or early November at the time of the first frost (Ishikawa et al., 2009). In winter, INA was maintained at high level. Interestingly, the flower bud scales of tropical *Rhododendron* species had very low INA irrespective of the season (Ishikawa et al., 2009). These authors surveyed the INA of various tissues of nearly 500 species, ranging from tropical to boreal plants and found that cold-hardy plants from temperate to boreal regions in general showed higher INA in some tissues compared with tropical plants. These results imply that when cold-hardy plants cold acclimate, they regulate freezing by producing intrinsic ice nucleators in tissues that undergo extracellular freezing or tissues that act as an ice sink in extraorgan freezing. It appears that tropical plants (sensitive to freezing) lack such



intrinsic ice nucleators and supercool extensively (lower than  $-7^{\circ}\text{C}$ ). When freezing does occur, it is lethal presumably due to intracellular freezing. The isolation and identification of such intrinsic ice-nucleating substances has not to our knowledge been successful. The level of supercooling goes down to  $-41^{\circ}\text{C}$  (Burke and Stushnoff, 1976) as the solid, denser form of water is the hexagonal ice having the structure that is uncanonical with respect to the pentagonal-like symmetry of a solidifying liquid that contains a high number of incommensurable nuclei of icosahedra and dodecahedra.

#### 11.1.4 ANTI-ICE-NUCLEATING ACTIVITIES

Anti-ice-nucleating activities can occur in three ways. (1) The different tissues have their specific INA, with or without specific ice-blockers. (2) Plants have barriers between organs and tissues to avoid spreading the ice nucleation (ice front) to other organs or to slow down spreading the ice front. (3) Third possibility is to produce specific ice-blocker which can inhibit INA of either plant intrinsic or extrinsic origin. The rate of ice propagation in veins was significantly higher at lower temperatures and reached to  $24\text{ cm s}^{-1}$  (Hacker and Neuner, 2008).

##### 11.1.4.1 Specific Ice Nucleation Activity

Since deep supercooled tissues such as florets and xylem ray parenchyma are too small, it is difficult to obtain a sufficient amount of these tissues (without contamination from other tissues) for biochemical analyses. To overcome this problem, Larcher et al. (1991) selected *Trachycarpus fortunei*, which is probably the most cold-hardy palm species and employs deep supercooling as the mechanism of cold hardiness in most of its leaf tissues. The leaf tissues of this species tolerated  $-14^{\circ}\text{C}$  without any injury. NMR micro-imaging with a resolution of  $31\text{ }\mu\text{m}$  successfully visualized non-invasively, the freezing behavior of *Trachycarpus* leaves. Water in the vascular bundles and epidermis froze at  $-10^{\circ}\text{C}$ , which corresponds to the higher temperature exotherm (HTE); however, the mesophyll cells remained deep supercooled to  $-14^{\circ}\text{C}$ . The cells on the adaxial side froze first, mostly between  $-16^{\circ}\text{C}$  and  $-19^{\circ}\text{C}$ , followed by freezing of cells on the abaxial side ( $-19^{\circ}\text{C}$  and  $-21^{\circ}\text{C}$ ). The hypodermis and vascular sclerenchyma remained deep supercooled to  $-22^{\circ}\text{C}$  or lower (Ishikawa et al., 2009). This implies that *Trachycarpus* leaves may have mechanisms to remain supercooled in a stable manner and/or to avoid the ice nucleation. At least several compounds are involved in the anti-nucleating activity but it still remains unknown whether they also confer supercooling stabilizing activity or not (Ishikawa et al., 2009; Wisniewski et al., 2009).

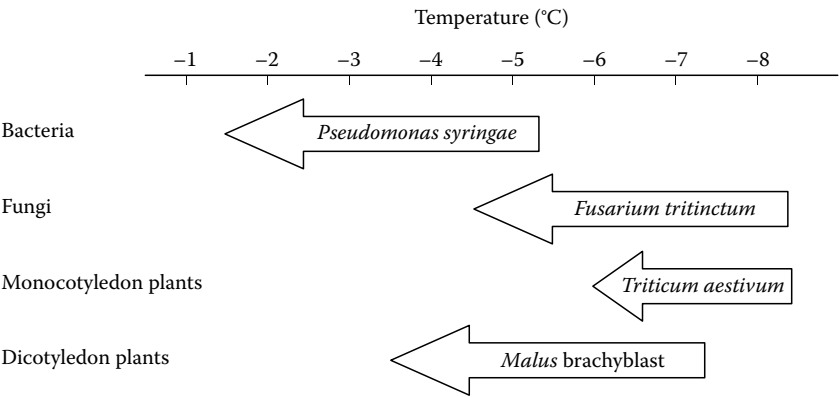
##### 11.1.4.2 Barriers to the External Ice Nuclei

The origin of external ice nuclei can recruit from dust, soil particles, and ice crystals, in case of biological ice nucleation from bacteria and fungi (Zámečník et al., 1991). A comparison of ice nucleation of bacteria cells and fungi spores, depending on their concentration with higher ice nucleation temperature, is shown in Table 11.2.

The presence of stomata, lenticels, and injured small surface trichomes or epidermal cell seems to be the gates for ice nucleation. Often, the growth of surface ice into the plant appears to be much less important than the spread of freezing throughout the plant from intrinsic nucleation events (Pearce, 2001; Wisniewski et al., 1997). New technique, high-resolution infrared (IR) thermography (Fuller et al., 2009; Gusta et al., 2004; Hacker and Neuner, 2007, 2008) has been used for thermal behavior of water during cooling the plants. By this technique, the ice nucleation induction and ice spreading through the plant have been studied more.

*Hydrophobic barriers to ice propagation* were shown clearly on rhododendron. Wisniewski et al. (1997) proved that the cuticle blocks an external ice as ice nuclei from inducing an internal nucleation event. Thick cuticle as a barrier to the ice propagation was also confirmed in cranberry. Wisniewski and Fuller (1999) demonstrated that ice crystals must physically grow through a crack in the cuticle of a non-tolerant plant, i.e. bean leaves (*Phaseolus vulgaris*), of broken epidermal hair, or of a stoma to induce ice nucleation within the leaf and the whole plant. From this it can be

**TABLE 11.2**  
**Example of Highest Nucleation Temperature for Bacterial Strain at Concentration  $10^8$  cfu mL<sup>-1</sup>, Fungi Spores in Concentration of  $10^5$  mL<sup>-1</sup>, Expressed Sap from Winter Wheat Crowns in Cold-Hardened Stage and Apple Brachyblast Collected from Tree in Mid-Winter Time**



*Notes:* Ice nucleation was measured in all samples with the same droplet freezing method by thermobattery in thirty 10  $\mu$ L drops at cooling rate 1°C min<sup>-1</sup>. Water for dilution was double distilled water with average nucleation temperature -18°C (Zámečník unpublished).

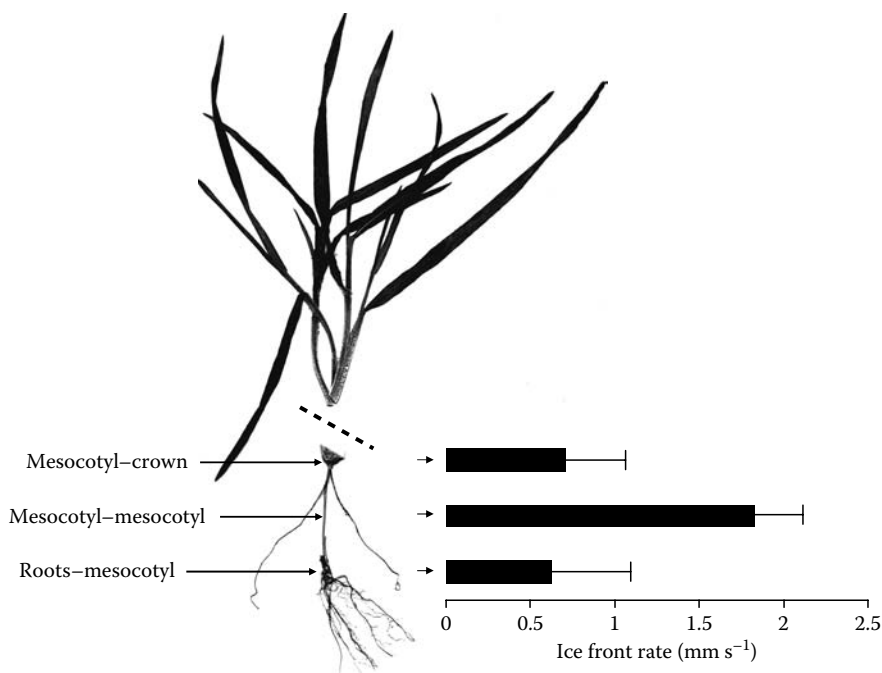
concluded that the cuticle acts as a primary barrier to the ice propagation from external sources of ice, probably due to their hydrophobic properties. Coated tomato (Wisniewski et al., 2002b) and potato plants (*Solanum tuberosum*) support this hypothesis. In the case of tomato, leaves coated with hydrophobic particle film have the temperature of ice nucleation 3.5°C lower than non-coated leaves sprayed with ice nucleation active (INA) bacteria (Wisniewski et al., 2002b).

**11.1.4.3 Barriers to Internal Ice Propagation**

Barriers to the spread of ice are common in woody plants, though often the nature or mode of functioning of the barrier is unclear. However, in forsythia and peach the growth of xylem into buds in the spring removes a barrier to ice (Ashworth et al., 1992). In plants there are also simple structures and specific macromolecules that appear to have a specific role in controlling nucleation of freezing or growth of ice (Zámečník, 1996). The supercooling of different plant parts could be important for survival and these barriers to ice propagation disappear due to seasonal pattern (Figure 11.4).

**11.1.4.4 Plant Structure and Its Role in Formation and Propagation of Ice**

It has been commonly observed that the formation of ice within a plant does not occur in a uniform manner but rather at selected sites where ice preferentially accumulates [reviewed by McCully et al. (2004) and Ishikawa et al. (2009)]. The factors associated with the determination of sites of ice formation and accumulation are not clearly understood. The mid-rib of leaves, the base of petioles, areas near vascular bundles, and phloem fibers all have been identified, using IR technology, as common sites for initial nucleation events. Ice nucleation in rape leaves that comes either from mid-vein, small veins, and/or mesophyll between veins (Zámečník, unpublished). Carter et al. (2001) and Fuller et al. (2009) have noted the existence of barriers within a plant that influence the direction and, thus, the rate of ice propagation. In some cases, this may directly affect the resulting pattern of injury that has occurred. It appears that where ice forms, how it propagates, and how it is accommodated are all important factors that affect the ability of a plant to survive freezing and may be as



**FIGURE 11.4** Harmonic mean with standard error of ice propagation rate in the equal distance of root-mesocotyl, mesocotyl-mesocotyl, mesocotyl-crown.

important as the ability to withstand the dehydration stresses associated with ice formation. Gusta et al. (2009b) conducted a detailed analysis of ice formation in the petioles of two frost-tolerant herbaceous plants. It was concluded that these plants have evolved a complicated arrangement of structural strengths and weakness within petiole tissues, which enables them to accommodate large volumes of intercellular ice during freezing events. These findings raised several questions regarding the composition and quality of the cell walls associated with the points of strengths and weakness, and the mechanism of water movement to and from the sites of ice formation.

#### 11.1.4.5 Specific Ice-Blocker

Boreal hardwood species had xylem parenchyma cells that adapt to subfreezing temperatures by deep supercooling. Crude extracts from xylem in all these trees were found to have anti-INA that promoted the supercooling capability of water. The magnitude of the increase in supercooling capability of water droplets in the presence of ice-nucleation bacteria, *Erwinia ananas*, was higher in the range from 0.1°C to 1.7°C on addition of crude xylem extracts than freezing temperature of water droplets on addition of glucose in the same concentration (100 mosmol kg<sup>-1</sup>) (Kasuga et al., 2007).

INA bacteria were also used for the detection of anti-nucleation activity of wheat and triticale seedlings. The ice nucleation activity of INA bacteria of *Pseudomonas syringae*, strain CCM 4073, was depressed by 1.1°C, with expressed sap from hardened winter-hardy wheat and triticale cultivars (Zámečník and Janacek, 1992). Consequently expressed sap from plants contains intrinsic ice nuclei that have no additional effect on ice nucleation of INA bacteria. The expressed sap from nonacclimated seedling of both species has no significant effect on the ice nucleation temperature of INA bacteria as well. Sun (2002) and Wisniewski et al. (2002a) indicated that by somehow blocking the activity of extrinsic nucleating agents, one may allow plant to supercool to a lower temperature and thereby provide some frost protection (Fuller et al., 2003). They used IR thermography to examine freezing in young tomato (Wisniewski et al., 2002b) plants and determine if a hydrophobic barrier on the plant surface prevents the action of extrinsic nucleating agents.

### 11.1.5 FROST DESICCATION/DEHYDRATION

The rate at which the volume of intracellular water changes with temperature was expressed by Mazur (1963) as follows:

$$\frac{dV}{dT} = \frac{kART}{V_i^o} \ln \frac{p_e}{p_i} \quad (11.12)$$

where

$k$  is the permeability constant of the cell  $\mu\text{m}^3$  water per  $\mu\text{m}^2$  cell membrane surface per minute per difference in water vapor pressure (between inside and outside the cell)

$A$  is the cell membrane area

$V_i^o$ , the molar volume of water

$p_i$  and  $p_e$ , the vapor pressures of supercooled water inside and at thermodynamic equilibrium outside the cell, respectively

$R$  is the gas constant

$T$  is the temperature

From this formula, the rate of water loss in extracellularly frozen cells, as the temperature decreases, is mainly determined by three parameters: (1) permeability constant, (2) surface area of the cells, and (3) difference in the vapor pressure between the supercooled water inside and over the ice outside the cells. The rate of diffusion of water to ice outside the cells is limited by the permeability of plasma membrane lipids. Therefore, if the temperature drops rapidly enough, the diffusion to the extraplasma-  
tic ice cannot occur with sufficient speed.

Woody plants tolerating ice crystals in their tissues have three strategies to survive temperatures below zero. The first living strategy is avoiding freezing by supercooling or lowering of melting point of their tissues and organs (Malone and Ashworth, 1991). Supercooling is a known status in many plants and plant tissues; in parenchymatic cells (Ristic and Ashworth, 1994) and in whole organs as generative buds of *Malus* (Bilavcik and Zámečník, 1996).

The second strategy of plants to stay alive after low temperatures thawing is tolerating of extracellular freezing. The survival of such plant cells/tissues is based on their tolerance to excessive dehydration of the protoplast. Some plant tissues survive temperatures even below  $-40^\circ\text{C}$  in the nature, and in the techniques used for preservation of plant genetic material they survive cryo-temperatures as low as  $-196^\circ\text{C}$ . Cell resistance to volume changes during extracellular freezing leads to cell tension during extracellular freezing. As a cold acclimation in leaves of many evergreen broadleaf species, supercooling stem xylem was associated with an increase in cell wall rigidity (Rajashekar and Lafta, 1996). Cold-hardy stem xylem developed cell tension as high as 27 MPa whereas cold-hardened leaves of live oak (*Quercus virginiana*) developed peak cell tension of 16.8 MPa during an extracellular freezing. The cell rigidity affects freeze-induced dehydration and the supercooling behavior of plant tissues. A relationship between bulk modulus and cell dehydration, including the extreme case where supercooling occurs, has been developed.

The third strategy of plants to survive frost is so-called extraorgan freezing (Sakai, 1982). By this type of strategy the whole plant organ such as bud is protected against ice nucleation and spreading, and the external ice formation exposes the bud to frost dehydration (Chalkerscott, 1992). The above-mentioned survival strategies of plant species tolerating ice in their tissues can be combined; for example, the woody plants can exhibit extraorgan freezing in their buds, extracellular freezing in their bark tissues, and supercooling in their parenchymatic cells of xylem rays. The question is what is the state of water in the plant cells subjected to low subzero temperature. The physical rule states that supercooled liquid changes to the glass at the glass-transition temperature ( $T_g$ ). Glassy state found in dormant twig of poplar tree (Hirsh et al., 1985) offers the explanation, in which state water can be found in frozen tissues. Authors suppose that this state occurred thanks to the freezing dehydration of tissues.

### 11.1.6 COLD ACCLIMATION

A common example of how single stress can increase resistance to other stress is how water deficit can increase of a single resistance to low temperatures. There are many other examples described in literature, for example, rye (Siminovitch and Cloutier, 1982) and wheat (Cloutier and Siminovitch, 1982). The short water deficit induces a dehydration and loss of turgor which by itself decreases ice formation after a subsequent low temperature stress (Palta, 1990).

The main handicap to active growth and metabolism at temperatures close to 0°C lies in the slowness of chemical reactions under these conditions, which can be overcome only by a correspondingly higher efficiency of all essential enzymatic mechanisms. This interesting aspect of the problem is largely unexplored.

Freezing damage is in general not a consequence of low temperature per se, but rather the result of cellular dehydration brought about by extracellular ice crystallization. Cellular membranes have been recognized as the primary sites of freezing injury. A *freezing tolerance* is defined as the ability of plants to survive ice formation in extracellular tissues without a significant damage of membranes or other cell components. It is the result of physiological, chemical, and physical consequences.

Furthermore, the exposure to frost after cold acclimation enhances the level of the freezing tolerance and of the gene expression (Pearce et al., 1998), indicating that the dehydrating effect of freezing might have a signaling role. Thus, a water-stress signal can be additional to or integral to the cold signal.

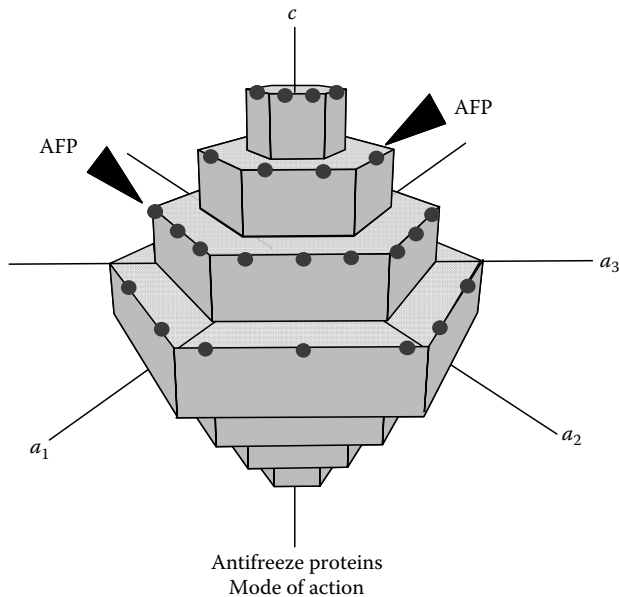
### 11.1.7 ANTIFREEZE PROTEINS

Published reports on Plant Antifreeze Proteins (AFPs) (proteins that bind to ice crystals and affect their morphology, and have the ability to inhibit nucleating compounds, exhibit hysteresis in very low extents unlike those found in fish and insects) and anti-nucleators (compounds that inhibit the activity of nucleating agents but do not exhibit hysteresis) have added to the complexity of our understanding of what induces a plant to freeze (Griffith et al., 2005; Griffith and Yaish, 2004; Ishikawa et al., 2009). There are many other peptides induced by low temperatures that play a crucial role during hardening but these are not connected with ice formation as the AFP. The AFPs are the only ones of the stress proteins about which the binding to ice crystals is well documented and, moreover, they have specific binding to different ice crystal planes. The plant AFP analysis is problematic, because their isolation is difficult due to their low abundance in the apoplast, in contrary to arctic fish (Figure 11.5).

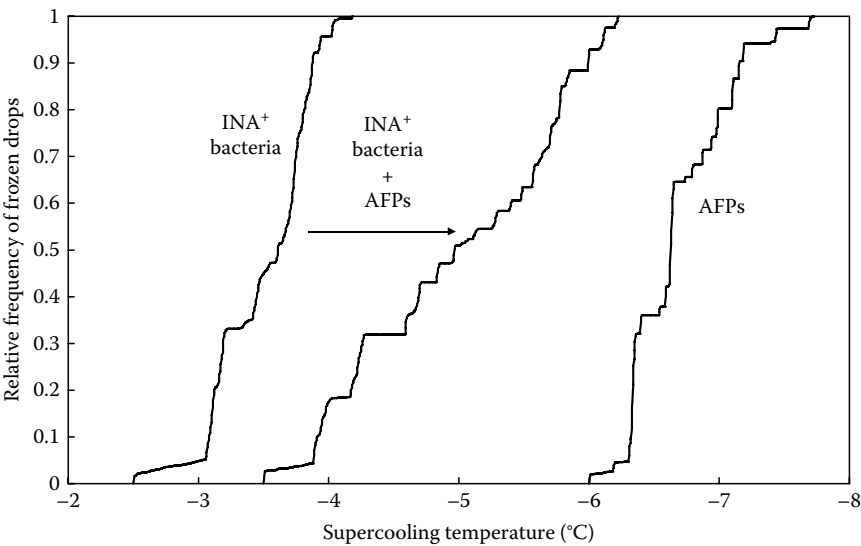
Griffith and coworkers (Hon et al., 1995) first reported that three Pathogenesis-Related (PR) proteins in the apoplast of cold-hardened winter rye have antifreeze activity. Antifreeze activity has been detected in the apoplast and also in triticale leaf and crown (Zámečník and Bieblova, 1994). Later, the antifreeze activity was also found at a polygalacturonase inhibitor homologue in carrot (Worrall et al., 1998; Meyer et al., 1999), a heat-stable protein in perennial ryegrass (Kuiper et al., 2001), a leucine-rich repeat-containing protein in wheat (Tremblay et al., 2005), and a WRKY protein in *Solanum dulcamara* (Huang and Duman, 2002).

The function of these AFPs in the apoplast of herbaceous plants which undergo extracellular freezing is not very clear, since the degree of thermal hysteresis (i.e., actual prevention of initiation of freezing) generated by these proteins is small. It is generally thought that they also inhibit the recrystallization of ice and thus maintain ice crystals in non-injurious small sizes during prolonged exposure to subzero temperatures, which may contribute to winter survival. (Zámečník et al., 2010) Wang et al. (2002) found that carrot AFP lost half of its recrystallization inhibition activity within 10–20 weeks at –80°C storage (Figure 11.6).

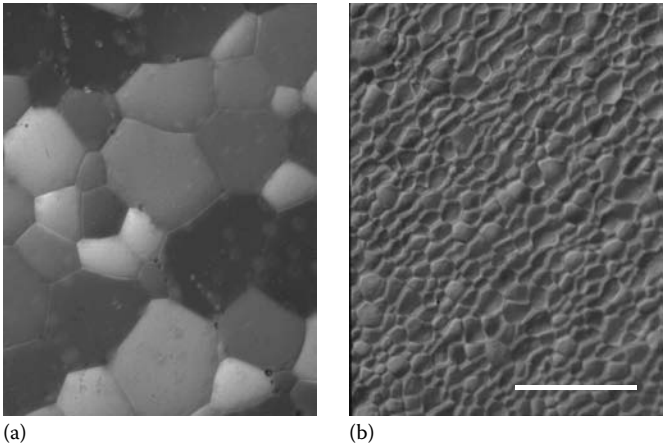
The presence of rye antifreeze activity has been shown to improve the freeze survival of non-acclimated ray suspension cells (Pihakaski-Maunsbach et al., 2003) and to influence freezing patterns of rye plants (Griffith et al., 2005). However, extracts from spruce, fir, and hemlock do not contain AFPs (Duman and Olsen, 1993); it might be that AFPs are not a universal component of freezing-tolerant plants (Figures 11.7 and 11.8).



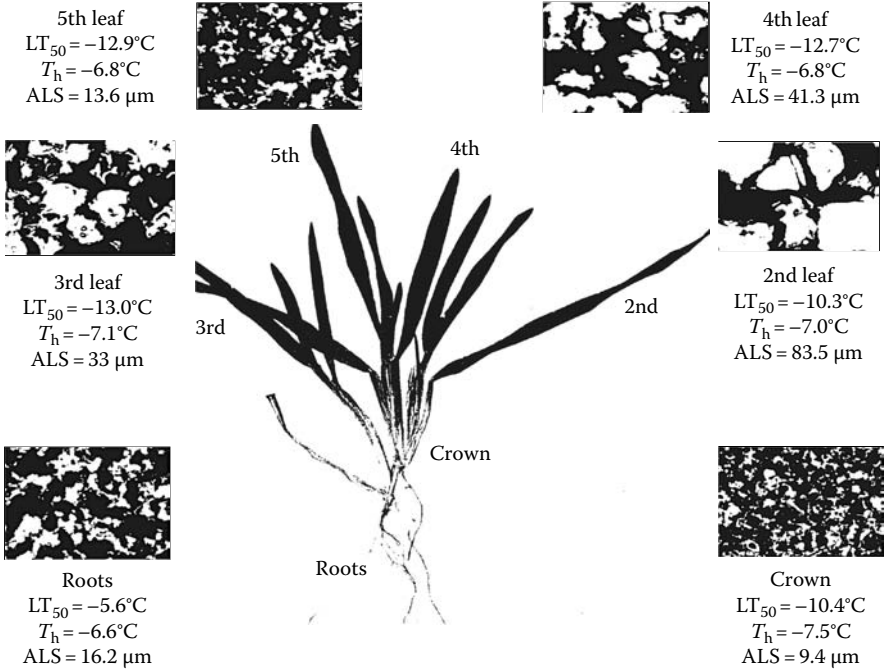
**FIGURE 11.5** Mode of action of antifreeze protein (AFP) binding on ice crystal. The proteins stop ice crystal growth in the  $a_1$ ,  $a_2$ , and  $a_3$  axes. Ice crystal growth is not so influenced in  $c$  axes, resulting in double spicks of ice crystal.



**FIGURE 11.6** Ice nucleation activity was (INA<sup>+</sup>) investigated by freezing droplet assay. Relative frequency of cumulative ice nucleation spectra of thirty 10  $\mu$ L drops. Nucleation event as a heat release of each drop was measured by thermobattery, with thermocouple in each drop in connection as differential thermal analysis. As a source of ice nucleation bacteria, *Pseudomonas syringae* strain CCM4073 in concentration  $10^7$  cfu with high nucleation activity ( $-3.6^\circ\text{C}$  of 50% frozen drops) and antifreeze proteins (AFPs) from extracellular washing of cold-hardened triticale seedling was used. Antifreeze proteins shifted nucleation temperature of bacteria after mixing with AFPs. *Note:* The absolute number of bacterial cells was equal in both samples (Zámečník and Janáček, unpublished).



**FIGURE 11.7** Ice crystals grown from distilled water undergoing recrystallization at  $-8^{\circ}\text{C}$ . (a) Recrystallization inhibition by antifreeze proteins from cold-hardened winter rye (cv. Musketeer) of  $1.18\text{ mg mL}^{-1}$ . (b) Bar is  $100\mu\text{m}$ . *Note:* Both figures are in the same magnification.



**FIGURE 11.8** Heterogeneity of frost resistance (expressed as lethal temperature  $LT_{50}$  after controlled freezing test in different low temperatures). Antifreeze protein activity (expressed as average linear size of crystals). Supercooling ability (measured by thermobattery, temperature was taken when a half of tested samples froze) of different organs of full cold-hardened winter wheat plant (Zámečník, unpublished).

### 11.1.8 WINTER HARDINESS

Tolerance to freezing temperatures is important in agriculture, where the ability to withstand late spring or early autumn frosts may determine the success of further growth and crop yield. In order to fully understand how plants are able to acclimate to low temperatures, it is helpful first to understand how and where freezing occurs in plants and how it causes damage.

The relative importance of stress factors causing winterkill can vary greatly among regions. In Ukraine, an analysis of data from the last 100 years showed that winterkill was caused by low temperatures in 35% of cases, by alternate freezing and thawing in 26% of cases, and by ice encasement in 22% of cases in the years when significant winter damage occurred. Poltarev et al. (1992) stated that the critical factors that affect winter survival in Poland are low temperature, freeze-induced desiccation, and infection by pathogenic fungi. Gusta et al. (1997) reported that the main factors responsible for winterkill in the Great Plains of North America are long periods of cold-induced desiccation, poor acclimation conditions in fall, and unpredictable timing and duration of extremely cold temperatures, whereas the primary cause of winterkill in western Canada is the freeze-induced desiccation. In the Czech Republic, the stability of frost resistance is of the main importance because the weather changed from maritime to continental and during winter all stresses caused by winter factors can occur.

Olien (1967) found that *winterkill* is most likely to occur during low-temperature stress following a midwinter thaw, when the crown tissues have a high moisture content. However, the basic process behind most events leading to winterkill is freezing or formation of ice in plant tissues. There are some stresses occurring during winter time in connection with water, no matter whether in excess or in shortage. Some of these stress factors can increase overwintering of the plant; the excess of water decreases the frost resistance and thanks to this percentage of overwintering plants decreases.

*Winter desiccation* can occur especially in the aboveground parts of the plants, which can dry gradually and die. Damage by dehydration occurs only at a high water saturation deficit (60%–90%). Disproportion between the uptake and output of water occurs because when the temperature falls below 0°C, the uptake of water by the roots is reduced drastically and when the soil freezes water uptake stops almost completely. Seedlings with root-freezing damage showed a reduction in survival and growth (Bigras and Dumais, 2005). Meanwhile, the aboveground parts of the plants continue to lose water even during the heaviest frosts (desiccation by frost) (Tahkokorpi et al., 2007). Transpiration is increased by low relative humidity, direct exposure to sunshine, and wind. Winter desiccation is particularly dangerous late in winter when the soil usually remains frozen and free of snow, while the air temperature increases above zero (Petr, 1991).

Plants are exposed to stress from *flooding* when the topsoil is no longer able to absorb the water after snow thawing or a long rain. Field observations suggest that flooding events in the growing season are more detrimental than in winter (Van Eck et al., 2005). Flooding is frequent in spring when the water from the thawing snow remains standing in the fields. Spring flooding can damage the plants directly and aggravate the damage which was caused by frost in winter.

If in a crop stand where water freezes or if water from melting ice freezes again, *ice sheets* develop. The survival of ice-encased plants depends on temperature, the porosity and thickness of the ice layer, the time for which the plants remain in the ice, and the frost resistance of the plants. The vitality of the plants declines markedly with the thickness of ice up to 20 mm; thereafter there is no decrease in vitality. Damage by ice encasement is ascribed to the toxic accumulation of CO<sub>2</sub> and ethanol which are the products of anaerobic respiration (Gudleifsson, 2009).

The hardening processes can be disturbed by a high soil temperature and by waterlogging of the topsoil. Excess water in topsoil also intensifies the frost injury. On the other hand, the hardening is encouraged if the plant water content is reduced by icy conditions. The trials of Cloutier and Andrews (1984) confirm that a drought stress has a positive influence on the induction of frost hardiness. In trials with winter wheat, water deficit alone partly hardened the plants to about 30% of the level of hardiness that would be induced by low temperature alone. A combination of low temperature with water deficit is the most effective treatment to raise hardiness (Willemot and Pelletier, 1979).

### 11.1.9 PLANT PROTECTION AGAINST LOW TEMPERATURES

There is a chronic and large demand for low cost, effective, and environmentally acceptable cold protection, also referred to as frost protection, techniques in temperate areas around the world to protect orchards (pome fruits, citrus, stone fruits, and nut crops), vineyards, cut flowers, and small



fruits (berries). The protection against freezing employs the unusual properties of water, especially its high heat capacity and high *exothermic heat* released during transition of water to the ice. The release of the latent heat of fusion (e.g., freezing water directly on plant-overtree sprinklers; or on surface under the canopy-undertree sprinklers) protect plants against freezing temperatures.

Most frost protection methods that increase the water vapor content of the air are generally beneficial. Heat from water is more efficient than some other sources because it is released at low temperatures. This is due to replacing radiation losses with the latent heat of condensation. A high dew point is powerful and effective mechanism selectively warms the coldest plant parts and is available for reducing freeze damage to plants.

Frost protection with water can require large volumes of water to be available for short periods of time, which often creates major physical and legal problems for growers. In addition, there may be other significant environmental consequences after thawing. Physically, there are problems because frost requires water deliveries before water is normally required for irrigation and many carrier systems are not ready for irrigation.

Protection against advective (windy) freezes is much more difficult to achieve than protection against radiative freezes. Consequently, most of the systems are practical and effective only under radiation situations. The formation of inversion layers is a benefit and many methods take advantage of an inversion to furnish, trap, and/or recirculate heat. There are many other methods used for protection of trees in the orchards and a combination of these increases the probability of surviving plants (e.g., mixing of the air, convective heating of the air release of the latent heat of condensation e.g., humidification, fogs, and sprinkler).

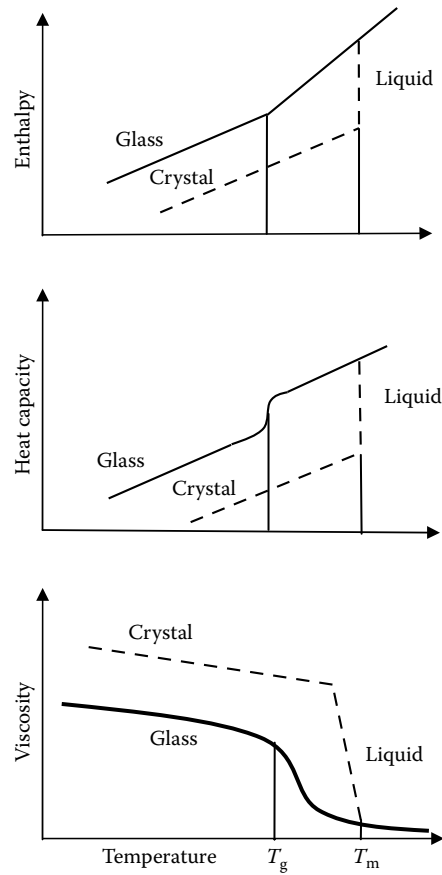
## 11.2 WATER FREEZING AT ULTRALOW TEMPERATURES

### 11.2.1 GLASS DEFINITION

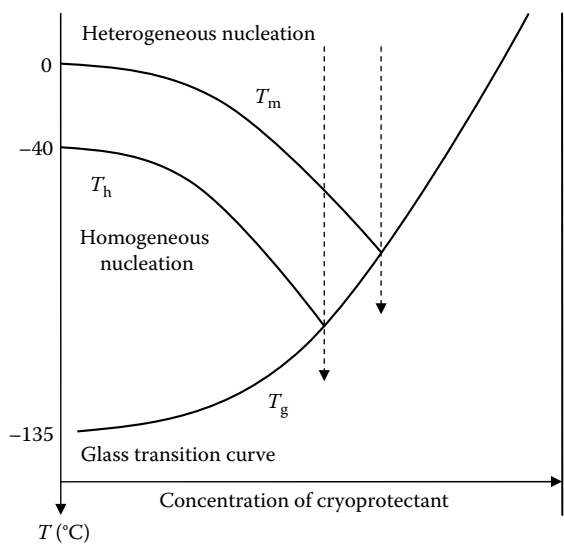
There is still much about the molecular physics and thermodynamics of glass that is not well understood, but we can give a general explanation of what is thought to be the case. Many solids have a crystalline structure on the microscopic scale. The molecules are arranged in a regular lattice. As the solid is heated, the molecules vibrate about their position in the lattice until, at the melting point, the crystal breaks down and the molecules start to flow. There is a sharp distinction between the solid and the liquid state that is separated by a *first-order phase transition*, that is, a discontinuous change in the properties of the material such as density. Freezing is manifested by a release of heat known as the heat of fusion (Figures 11.9 and 11.10).

Usually, when a liquid is cooled to below its freezing/melting point, crystals form and it solidifies; but sometimes it can become supercooled and remain liquid below its melting point because there are no nucleation sites to initiate the crystallization. If the viscosity rises enough as it is cooled further, it may never crystallize. The viscosity rises rapidly and continuously, forming thick syrup and eventually an amorphous solid. The molecules then have a disordered arrangement but sufficient cohesion to maintain some rigidity. This state is often called an amorphous solid or glass. In biological object we call it the biological glass (Buitink, 2000).

The vitrified state is metastable, which means that it can relatively easily revert back to a liquid and/or cold crystallize to form ice. Systems are particularly unstable during rewarming. Assigns in molecular mobility and energy may be sufficient to allow water molecules to rearrange (relax) and form ice. Vitrification can be achieved in a number of ways but all usually have the end results of increasing solute concentration to a critical viscosity. The viscosity of water at room temperature is about 0.001 Pa s. Thick oil might have a viscosity of about 0.1 Pa s. As a liquid is cooled, its viscosity normally increases, but high viscosity also has a tendency to prevent crystallization. For temperature-induced glasses, the point at which this occurs is called the glass-transition temperature ( $T_g$ ); molecular motion nearly ceases and the liquid becomes a glassy solid.



**FIGURE 11.9** Enthalpy, heat capacity, and viscosity change of liquid, glass, and crystal with temperature. On crystallization, the viscosity changes abruptly from  $\sim 100$  to  $\sim 10^{20}$  Pa s. A solid can be defined as material with a viscosity greater than  $10^{11}$  Pa s.



**FIGURE 11.10** Schematic state diagram of aqueous solution of cryoprotectant.

Glass is actually a supercooled liquid because there is no first-order phase transition as it cools. In fact, there is a *second-order phase transition* between the supercooled liquid state and the glass state, so a distinction between crystallization and glass transition can still be drawn (Zámečník et al., 2003). The transition is not as dramatic as the phase change that takes from liquid to crystalline solids. There is no discontinuous change of density and no latent heat of fusion. The transition can be detected as a marked change in the thermal expansivity and change of heat capacity of the matter. Heat capacity shows a step change at a second-order transition temperature. The glass transition has properties of a second-order transition, but the transition is not well-defined and it occurs over a temperature range.

The temperature at which the glass transition takes place can vary according to how slowly the plant material cools. If it cools slowly it has longer time to relax, the transition occurs at a higher temperature and the glass formed is more dense. If it cools very slowly, it crystallizes, so there is a minimum cooling rate for the glass formation.

A liquid to crystal transition is a thermodynamic; that is, the crystal is energetically more favorable than the liquid when it is below the melting point. The glass transition is purely kinetic, that is, the disordered glassy state does not have enough kinetic energy to overcome the potential energy barriers required for movement of the molecules. The molecules of the glass take on a fixed but disordered arrangement. Glasses and supercooled liquids are both metastable phases rather than true thermodynamic phases like crystalline solids. In principle, a glass could undergo a spontaneous transition to a crystalline solid at any time.

The hypothesis that the glassy states are results of change from being a supercooled liquid to an amorphous solid at the glass-transition temperature, but it is difficult to justify. Polymerized materials such as rubber show a clear glass transition at low temperatures but they are normally considered to be solid in both the glass and rubber conditions. Glass is defined sometimes as being neither a liquid nor a solid. It has a distinctly different structure with properties of both liquids and solids (Figure 11.1).

Viscosity increases rapidly to solidification near  $T_g$ , but over a small temperature range rather than at a precise temperature (in contrast to fusion, which occurs at a precise temperature). The change that happens at  $T_g$  is a rapid increase in viscosity but not a change of state. Viscosity becomes very high near  $T_g$  when cooling from above, which means that  $T_g$  is better characterized as a “rubber/glass transition” than a “liquid/glass transition.” In practice,  $T_g$  occurs within a narrow temperature range because changing a cooling rate an order of magnitude (i.e., by a factor of 10) only changes  $T_g$  by 3°C to 5°C. The situation at the level of molecular physics can be summarized that there are four main types of molecular arrangement of water: water as a liquid, water in vapor phase, as a solid (ice), and finally as a glass (Figure 11.1).

### 11.2.2 PHYSICAL CHEMISTRY OF VITRIFICATION

Three phenomena are necessary for glass transition involvement: dehydration, quench freezing, and quench warming. Water is not very viscous; therefore, it can be vitrified only by an extremely rapid quench freezing of a small sample. Under such rapid cooling, water molecules do not have time to arrange themselves into a crystalline lattice structure. Viscosity increases very little when water is cooled, but at freezing temperature a sudden phase transition occurs to an ice crystal.

In contrary, volume continues to decrease and viscosity continues to increase below  $T_g$ . Because cooling occurs from outside to inside, overlay rapid cooling creates stress when the warmer core needs to contract more than the cooler surface. This may be the reason why slow cooling reduces cracking. Plants are sometimes tearing in to small pieces by cracking. At  $T_g$  there is a sudden increase in viscosity and heat capacity (usually many orders of magnitude), but there is no comparable sudden decrease in volume. The Kauzmann temperature ( $T_k$ , ideal glass-transition temperature) is a hypothetical temperature at which the configurational entropy of a supercooled liquid is presumed to be reduced to that of a crystalline state (Figure 11.9).

11.2.3 CRYOPRESERVATION METHODS

If the freezing rate of cells or tissues is sufficiently slow, less than  $10^{\circ}\text{C min}^{-1}$ , ice is form outside the protoplasts where water is purest. Protoplasmic water will migrate out of the cells and add to the extracellular ice crystals. Extracellular ice does not kill plant cells and the tissues can normally be rewarmed without injury. At intermediate freezing rates ( $10\text{--}100^{\circ}\text{C min}^{-1}$ ), ice crystals are formed within the protoplasts. Intracellular ice formation disrupts the fine structure of the cells and invariably results in death.

How rapidly should plant samples be cooled from  $10^{\circ}\text{C}$  to  $-196^{\circ}\text{C}$ ? Pure water can be vitrified by cooling it at a rate of about 3 million  $^{\circ}\text{C}$  per second to about  $-138^{\circ}\text{C}$ , but this cooling rate is impractical for cryopreservation. The rate of cooling required to produce a vitrified (glassy) state is slower for solutions containing cryoprotectant than for pure water. The rate of cooling which produces glass state is referred as the critical cooling rate.

Vitrification occurs in cells of twigs of some woody plants such as poplar, which could explain why twigs of several woody species can survive the temperature of liquid nitrogen (Sakai, 1960). These findings of biological object vitrification gave a background for further cryopreservation of plants not tolerating extracellular freezing of water (Reed, 2008) (Table 11.3).

Completely ice-free cryogenic storage is the latest approach to plant cryoconservation. The key to developing cryoprotective vitrification strategies is to increase cell viscosity to the point at which ice formation is inhibited and water becomes vitrified after exposure to cryogenic temperatures.

Plantlets may be preconditioned in special media or with specific growth conditions, precultured with cryoprotective chemicals just prior to cryopreservation, and protected with vitrification solutions prior to plunging in to liquid nitrogen.

For glass detection, it is usually used differential scanning calorimeter (DSC) which is used for the direct estimation of glass-transition temperature, ice nucleation temperature, or melting temperature and amount of freezable water (osmotically active water) in a cell or tissues and the percent of the cell volume which is osmotically inactive. Estimate the osmotically inactive fraction of water by the Boyle–van’t Hoff plot (Sun, 1997), which plots a cell volume as the dependent variable and a reciprocal of external osmolality as the independent variable.

Glass-transition temperature is related to the value of  $T_m$  on a Kelvin temperature scale for substances that vitrify:

$$T_g = \frac{2}{3T_m} \tag{11.13}$$

On the Kelvin temperature scale, it is a rule of thumb that  $T_g$  occurs at about two-thirds the value of the melting temperature ( $T_m$ ). Substances for which  $T_g/T_m$  is significantly greater than  $2/3$  are *fragile* (according to the terminology of Angel) (Hancock and Shamblin, 2001). Liquids classified

**TABLE 11.3**  
**State of Water-Influenced Physiological Process during Cryoprotocol**

| Temperature | Water-Dependent Process | Physiological Process                      |
|-------------|-------------------------|--------------------------------------------|
| Pre-cooling | Dehydration stress      | Water removing, osmotic potential decrease |
| Cooling     | Supercooling            | Separation of ice nuclei                   |
|             | Osmotic stress          | Plasmolysis                                |
|             | Desiccation stress      | Solute concentration                       |
|             | Glass transition        | Biological glass formation                 |
|             | Osmotic stress          | Deplasmolysis                              |
| Warming     | Rehydration             | Water equilibrium, growth                  |

as “strong glass formers” show only a small change in viscosity at  $T_g$ , whereas so-called “fragile” liquids such as polymers show very large changes in viscosity at  $T_g$ .

Generally, the glass-forming substances can be divided according to nucleation and growth control as follows:

Poor glass formers:

1. Liquids that quickly form large numbers of nuclei close to  $T_m$ .
2. Crystals that grow very quickly.

Good glass formers:

1. Liquids that are too sluggish to form nuclei even far below  $T_m$ .

In the case of dehydrating processes, water is removed by osmotic dehydration, freeze drying, and/or by the evaporation of water to outside the cells and tissues. This is done mainly by using more concentrated cryoprotectants (mainly mixture of penetrating and non-penetrating cryoprotectants and by slow cooling rate and/or by dehydration in airflow in flow boxes or over a silica gel. Using this method for cryopreservation, other problems arise: sensitivity to cooling rate, desiccation sensitivity rate, and final value of dehydration desiccation sensitivity. The desiccation sensitivity does not depend only on sensitivity to a different rate of desiccation but also on the final level of dehydration. Minimum water or nonfreezing water volume is called “vital” water.

#### 11.2.3.1 Two-Step Freezing Method

In two-step freezing experiments, believed to mimic dehydration stress, protoplasts can undergo reversible contraction-expansion cycles, or osmotic excursions, when slowly cooled and warmed from  $>0^\circ\text{C}$  ( $\psi_w \sim -0.5\text{ MPa}$ ) to temperatures of  $-5^\circ\text{C}$  to  $-20^\circ\text{C}$  ( $-2.5 \geq \psi_w \geq -6\text{ MPa}$ ). A 60%–80% reduction in cell volume occurs when the water potential of cells decreases from about  $-0.5\text{ MPa}$  to about  $-4.5$  to  $-6\text{ MPa}$  ( $-4^\circ\text{C}$  to  $-5^\circ\text{C}$ ) (Meryman, 1974; Steponkus and Lynch, 1989).

Two-step freezing method is mainly used in two steps with different cooling rates (Reed and Uchendu, 2008). In the first step of this method, the plants are cooled to a certain temperature, usually to  $-40^\circ\text{C}$ , by the rate ranging from  $0.25^\circ\text{C min}^{-1}$  for mint to  $2^\circ\text{C min}^{-1}$  (Towill, 2002). Using cell suspension cultures of *Puccinellia distans* derived from callus, it was found that the cooling rates were below  $1^\circ\text{C min}^{-1}$  (Heszky et al., 1990). After reaching desired temperatures (from  $-30^\circ\text{C}$  to  $-40^\circ\text{C}$ , the usual terminal temperature  $-40^\circ\text{C}$ ), the samples are cooled much faster till the liquid nitrogen temperature is reached. The cooling rate in the second part of slow freezing method was as high as possible.


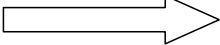
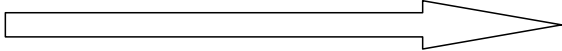
If cooling is too slow, however, cells will be killed by prolonged exposure to low temperature. During the first part of this method time is given to remove water. *Two-step freezing method* is based on slow cooling to a subzero temperature, usually to  $-40^\circ\text{C}$ . During this equilibrium freezing, osmotic equilibrium is established, and then followed by rapid cooling to the liquid nitrogen temperatures. By slow cooling the cells can be killed either by toxic concentrations of electrolytes that form inside the cell or by mechanical crushing from extracellular ice.

#### 11.2.3.2 Cryoprotectants

A colligative property of a solution depends only on the ratio of the number of particles of solute and solvent in the solution, not the identity of the solute. The theory of colligative effect has two essential attributes (Benson, 2008):

1. Cryoprotectants must be able to penetrate the cell, or else they will cause osmotic dehydration and cause the very injury they are used to protect against.
2. Cryoprotectants must be nontoxic to the cell at the concentrations required for their efficacy. Penetrating cryoprotectants make a contribution to the overall osmolality of the cell (Table 11.4).

**TABLE 11.4**  
**The Classification of Main Cryoprotectants according to Their Penetration to the Cells**

| Group of Cryoprotectants                                 | Intercellular Space                                                                | Cell Wall | Plasmatic Membrane | Cytoplasm |
|----------------------------------------------------------|------------------------------------------------------------------------------------|-----------|--------------------|-----------|
| Soluble proteins, polysaccharides, PEG 6000 <sup>a</sup> |   |           |                    |           |
| Sucrose, manitol, prolin, PEG 1000 <sup>a</sup>          |   |           |                    |           |
| Glycerol, DMSO <sup>b</sup>                              |  |           |                    |           |

Source: Adapted from Tao, D.L. and Li, P.H., *J. Theor. Biol.*, 123, 305, 1986.

<sup>a</sup> Polyethylene glycol, with molecular weight.

<sup>b</sup> Dimethylsulfoxide.

The glass-transition temperature ( $T_g$ ) is important for cryopreservation of the cryoprotectant/water mixture. For most used cryoprotectants, the glass-transition temperatures as well as critical concentrations are given in Table 11.5. It is possible to divide the cryoprotectants to penetrating and non-penetrating cell wall and plasmalemma (Tao and Li, 1986). Penetrating cryoprotectants have a colligative property in which the salt is buffered down to low temperatures. They are able to cross the cell wall and cell membrane and they buffer the intracellular salt as well.

Non-penetrating cryoprotectants are thought to act by dehydrating the cell at high sub-freezing temperatures. These cryoprotectants help for rapid cooling and glassy state involvement. These compounds are specific with their high molecular weight (generally polymers) that form extensive hydrogen bonds with water, reducing the water activity to a much greater extent than would be predicted by their molar concentration (they do not obey Raoult's law). See the list in Table 11.5 of the main cryoprotectants.

**TABLE 11.5**  
**Glass Transition of Cryoprotectants at Concentration of Cryoprotectants Required to Vitrify**

| Cryoprotectant    | Concentration for Vitrification % (w/w) | Glass Transition (°C) |
|-------------------|-----------------------------------------|-----------------------|
| Sucrose           | 63                                      | -86                   |
| Glycerol          | 65                                      | -114                  |
| Ethylene glycol   | 55                                      | -131                  |
| DMSO <sup>a</sup> | 49                                      | -137                  |
| Propylene glycol  | 44                                      | -109                  |

<sup>a</sup> Dimethylsulfoxide.

### 11.2.3.3 Plant Vitrification Solution

Vitrification solutions involving vitrification were developed in the 1990s (Langis and Steponkus, 1990; Sakai et al., 1990), and the number of cryopreserved species has increased markedly since then. In vitrification procedures, cells and meristems must be sufficiently dehydrated before they are plunged in liquid nitrogen (Langis and Steponkus, 1990). Sakai (1960) used glycerol-based Plant Vitrification Solutions designated 2 (PVS2). PVS2 contains 30% glycerol (w/v), 15% ethylene glycol (w/v), and 15% dimethylsulfoxide (DMSO; w/v) in basal culture medium (without growth regulators) containing 0.4 M sucrose (pH 5.8). Nishizawa et al. (1993) developed Plant Vitrification Solution No. 3 (PVS3) with composition of 50% glycerol (w/v) and 50% sucrose (w/v) in water.

PVS2 easily supercools below  $-100^{\circ}\text{C}$  upon rapid cooling and solidifies into a metastable glass at about  $-115^{\circ}\text{C}$ . Upon subsequent slow warming, plant have a glass-transition temperature ( $T_g$ ) at about  $-115^{\circ}\text{C}$ , with an exothermic cold crystallization at about  $-75^{\circ}\text{C}$  and an endothermic melting  $T_m$  at about  $-36^{\circ}\text{C}$ .

In brief, the cryopreservation procedure has the following steps (Sakai et al., 2008):

1. Preculture of excised meristems on solidified medium with 0.3 M sucrose at room temperature or at  $0^{\circ}\text{C}$ .
2. Osmoprotection with loading solution. Loading with a mixture of 2 M glycerol and 0.4 M sucrose for 20–30 min at  $25^{\circ}\text{C}$ . An osmotic loading treatment increases the osmolarity of the cell and minimizes osmotic damage caused by the vitrification solution.
3. Dehydration with a vitrification solution (PVS2 or PVS3).
4. Rapid cooling/rapid warming.
5. Unloading, dilution of the vitrification solution.

It is evident from these steps that the water relation of cells and tissues is basic for the success of cryopreservation.

### 11.2.4 DEVITRIFICATION

*Devitrification* is interpreted as the transition of a glass into liquid. Sometimes the term devitrification is used for a different type of behavior; it is used for crystallization of water after crossing the glass-transition temperature. For this phenomenon, the usually used term is cold crystallization (Sikora et al., 2007). During warming of rapidly cooled cells, at low temperatures above the glass-transition temperature, the gradient in osmotic pressure driving water from the cell remains. Therefore, the conditions for membrane damage due to water flux persist during warming until the melting of ice reduces and then reverses the movement of water across the membrane. There is evidence that plasma membrane damage due to excessive osmotic pressure creates holes in the membrane, allowing ice to form intracellularly (Muldrew and McGann, 1994). However, warming from the glassy state to temperatures above the  $T_g$  results in a tremendous increase in diffusion, not only from the effects of the amorphous to viscous liquid transition but also from increased dilution as melting of small ice crystals occurs almost simultaneously ( $T_g = T_m$ ). The time scale of molecular rearrangement continually changes as the  $T_g$  is approached, so that food technologists can also gain some enhanced stability at temperatures above  $T_g$  by minimizing the  $\Delta T$  between the storage temperature and  $T_g$ , either by reduced storage temperatures or enhanced  $T_g$  through freezing methods. Hence, knowledge of the glass transition provides a clear indication of molecular diffusion and reactivity and, therefore, ideal state for a long-time saving of biological material in relative stable state.

### 11.2.5 GLASS STABILITY

Glassy status as a common solid/rigid state occurring in biological samples habitually results from a suitable circumvention of crystallization. The quality of glassy state is possible to define simply by ratio ( $T_g, T_m$ )-the lower the ratio, the higher the difference of  $T_g$  and  $T_m$ , a greater stability of glassy

state. A more sensitive interrelation to the glass formation peculiarities can be found on the basis of Hruby coefficient,  $K_g$

$$K_g = \frac{T_c - T_g}{T_m - T_g} \quad (11.14)$$

where  $T_c$ ,  $T_g$ , and  $T_m$  are temperatures of crystallization, glass transition, and melting temperature, respectively. It is clear that the greater the value of  $K_g$ , the better the glass forming. The best utilization of  $K_g$  is in the comparison of glass-forming ability and glassy-state stability of different biological materials under different conditions and thermal treatments. Hruby coefficient ( $K_g$ ) indicates the glass stability against crystallization on heating and can be used to estimate the vitrification ability of glass-forming liquids. Both of these characteristics ( $T_g$ ,  $T_m$ ) are easily measured by conventional differential thermal analysis or DSC during warming samples in glassy state.

The kinetic stability of biological glasses is clearly  $T_g$ -dependent. The reaction rate constants deviate notably from the traditional Arrhenius behaviors and can be well fitted to the Williams–Landel–Ferry (WLF)

$$\log a(T) = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)} \quad (11.15)$$

where  $C_1$  and  $C_2$  are universal WLF constants. It was found that different polymers exhibit very similar WLF constants. The universal values of constants in the WLF equation, used for a broad spectrum of amorphous materials ( $C_1 = 17.44$ ,  $C_2 = 52.1$ ) do not match to biological objects, however, to the dynamic processes of biological material. The derived  $C_1$  and  $C_2$  constants of the WLF equation for seed survival of three species at  $T > T_g$  are: for *Glycine* ~37.5 and 269.8, for *Pisum sativum* 22.3 and 179.2, and for *Phaseolus vulgaris* 22.1 and 183.1, respectively (Sun, 1997).

### 11.2.6 DIFFERENTIAL SCANNING CALORIMETRY

For thermal studies of plant samples in low and ultralow temperatures, Differential Thermal Analysis (DTA) is used. By this analysis, it is possible to measure the difference in temperature between, for example, wet sample and dry sample, to determine beginning of freezing. Thermal analysis uses Differential Scanning Calorimeter (DSC) with its modification as modulation of temperature. By this technique, it is possible to measure heat flow (Figure 11.11)

$$\frac{dQ}{dt} = \frac{C_p dT}{dt + f(t, T)} \quad (11.16)$$

where

$dQ/dt$  is total heat flow ( $W\ g^{-1}$  or  $J\ g^{-1}\ s^{-1}$ )

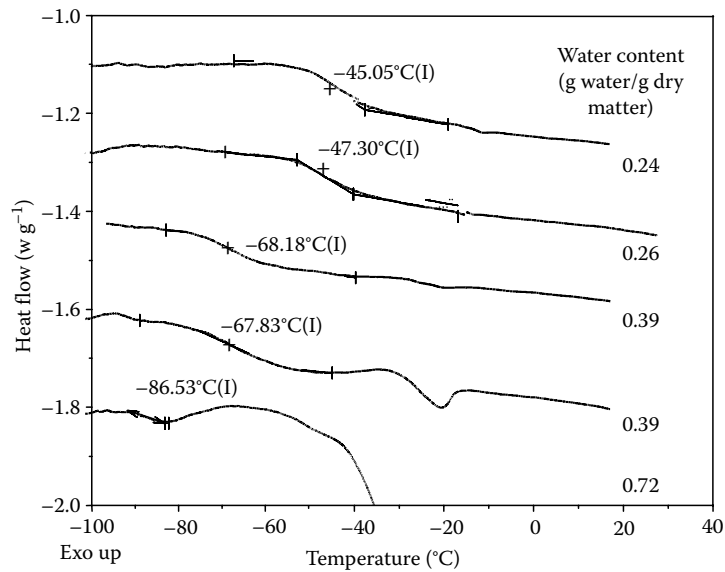
$C_p$  is specific heat capacity ( $J\ g^{-1}\ ^\circ C^{-1}$ )

$dT/dt$  is heating rate ( $^\circ C\ s^{-1}$ )

$f(t, T)$  is time-dependent response

DSC is used as a main tool in cryobiology to assist cryopreservation protocol development, to control and document cryoprotocols in usage, and to keep information about stored samples and their thermal properties (Zámečník and Šesták, 2009; Zámečník and Šesták, 2010). Water-state





**FIGURE 11.11** Shift of the glass-transition temperature to the higher temperature after dehydration of apple buds (Bilavčík, unpublished).

transitions were detected during the cooling and heating of samples as a function of time and temperature. The main two characteristics: the temperature of glass transition and the amount of frozen water are crucial for cryopreservation (Benson, 2008) (Table 11.6).

**Cryobanking.** The stress physiology of water behavior at low and mainly at ultralow temperature act as put on the vegetatively propagated plants, recalcitrant seeds, and pollen. There are many cryobanks round the world and in comparison to the cryobanking of other biological objects (medical and animal cryobanks), the plant cryobanking is the youngest of all of them.

**TABLE 11.6**  
**Comparison of Cold Tolerance of Shoot Tips Expressed as Lethal Temperature of Regrowth after Low and Ultralow Temperature Treatment**

|        | Cold<br>Acclimated | Slow Cooling          |                     |                           | Quench Cooling        |                     |                           |
|--------|--------------------|-----------------------|---------------------|---------------------------|-----------------------|---------------------|---------------------------|
|        |                    | LT <sub>50</sub> (°C) | T <sub>g</sub> (°C) | H g H <sub>2</sub> O/g DW | LT <sub>50</sub> (°C) | T <sub>g</sub> (°C) | H g H <sub>2</sub> O/g DW |
| Potato | N-CA               | -3.5                  | —                   | 4.8                       | <-196                 | -75 to -80          | 0.48                      |
| Hop    | N-CA               | -5                    | —                   | 6.1                       | —                     | —                   | —                         |
|        | CA                 | -10                   | ND                  | 4.5                       | >-196 (45%)           | —                   | 0.5                       |
| Apple  | N-CA               | -5                    | —                   | 5                         | —                     | —                   | —                         |
|        | CA                 | -20                   | ND                  | 3.8                       | <-196 (90%)           | -47 to -54          | 0.3–0.26                  |
| Garlic | N-CA               | -13                   | —                   | 2.1                       | —                     | —                   | —                         |
|        | CA                 | -16                   | -76                 | 2                         | >-196 (48%)           | -20                 | 0.2                       |

*Notes:* Slow cooling rate was 9°C min<sup>-1</sup> after ice seeding at -3°C, quench freezing rate was several orders higher after directly plugging the shoot tip meristems into liquid nitrogen (-196°C) with dehydration pretreatment. CA, cold-acclimated; N-CA, non-cold-acclimated plants; LT<sub>50</sub>, lethal temperature of survival/regeneration after freezing test; T<sub>g</sub>, glass-transition temperature measured by differential scanning calorimetry; H, hydration of shoot tips expressed as the ratio of weight of water to dry weight of plant matter after drying the samples at 105°C (Zámečník, Faltus, and Bilavčík, prepared for publishing); ND—not detected.

### 11.3 CONCLUSION

Plants, during evolution, have developed many mechanisms for how to survive low temperatures. Most of them tolerate high amounts of water in frozen state. Plant-stress physiology at low temperatures describes many mechanisms how plants can avoid, tolerate, or resist all states of water (supercooled water, ice and water in glassy state). Plants combine different strategies to survive low temperature even in one organ. In plant physiology of low-temperature stress, there is still a great amount of unanswered questions. Those that have been answered have a great influence either in technological protection of plants against low-temperature stress or genetic improvements of their cold tolerance.

Plant vitality, after storing them at ultralow temperature, is important for safe biodiversity keeping. Knowledge of water behavior at ultralow temperatures can help plants to survive from “outside.” Many modern cryopreservation methods are based on vitrification. Low water content is the main prerequisite for involvement in the glassy state in plant cytoplasm. On the other hand, the level of dehydration is closed to a vital amount of water and in antithesis in some cases when plants are mildly damaged by dehydration with the aim to increase regeneration after the cryopreservation protocol. From this point of view, the resistance to dehydration is very important for higher regeneration of thawed plants from ultralow temperatures.

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## *Part III*

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*Plant and Crop Responses:  
Physiology, Cellular, and Molecular  
Biology, and Microbiological Aspects  
under Salt, Drought, Heat, Cold,  
Light, and Other Stressful Conditions*



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# 12 Germination of Seeds and Propagules under Salt Stress

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## 12.1 INTRODUCTION

Germination of seed and propagules is a complex phenomenon involving many physiological and biochemical changes prior to activation of the embryo and protrusion of the radicle (Bewley and Black 1985, Mayer and Poljakoff-Mayber 1989, Bradford and Nonogaki 2007). During initial stages of germination, propagules, being vegetative tissues, may respond differently to seeds; however, fundamentally, seedlings from both grow similarly. Any unfavorable changes may jeopardize the process of germination (Espinar et al. 2005, Khan and Weber 2008, Wahid et al. 2008).

Salinity, as an abiotic hazard, induces many disorders in seeds and propagules during germination and/or sprouting (Koyro and Eisa 2008, Rasheed 2009). A negative correlation between salinity of substrate and seed germination/vigor has been well established (Rehman et al. 2000). Salinity arrests germination at higher levels and induces a state of dormancy at lower levels (De Villiers et al. 1994, Khan and Weber 2008). Salinity affects imbibition of water due to lowered osmotic potential of germination media (Bliss et al. 1986, Poljakoff-Mayber et al. 1994, Khan and Weber 2008). It also causes toxicity; that is, it changes the activity of enzymes of nucleic acids metabolism (Gomes Filho and Sodek 1988, Guerrier 1988, Gomes Filho et al. 2008), alters protein metabolism (Yupsanis et al. 1994, Dell'Aquila and Spada 1993, Dantas et al. 2007), upsets plant growth regulator balance (Khan and Rizvi 1994), and reduces the utilization of seed reserves (Mondal et al. 1988, Promila and Kumar 2000, Othman et al. 2006). It may cause changes at ultrastructural (Bliss et al. 1986, Koyro 2002), cell and tissue (Valenti et al. 1992, Wahid et al. 1998, Rasheed 2009), and organ levels (Reinhardt and Rost 1995, Rasheed 2009).

Salinity interacts with certain plant and environmental factors during germination. The main plant factors are the seed coat and other sterile seed structures (Eschie 1995, Ahmad et al. 2005, Kaya 2009), dormancy (Khan and Weber 2008), seed age (Smith and Dobrenz 1979, Khajeh-Hosseini et al. 2002, 2003), seed polymorphism (Khan and Ungar 1984a, Khan and Weber 2008), and seedling vigor (Rogers and Noble 1991, Lin and Kao 1995, Kaya and Day 2008). Among environmental factors, temperature (Khan and Ungar 1997a), light (De Villiers et al. 1994), availability of water (Hegarty 1978), oxygen (Wijte and Gallagher 1996a), and influence of other gases (Xu et al. 2006, Zheng et al. 2009) affect seed germination under saline conditions.

This chapter covers some physiological and biochemical aspects of seed germination and propagule sprouting under salinity. Internal (plant) and external (environmental) factors influencing germination of seeds and propagule sprouting in saline media are also discussed.

## 12.2 PROCESS OF GERMINATION UNDER SALINITY

Germination of a viable seed or propagule starts with imbibition of water and terminates with emergence of embryonic tissues. It involves the hydration of proteins, subcellular structural changes, respiration, synthesis of macromolecules, and cell elongation (Bewley and Black 1985, Bradford and Nonogaki 2007). Chong and Bible (1995) regarded growth of embryonic tissues as an important step in the completion of germination, while others emphasized seedling establishment under stressful conditions (Stumpf et al. 1986, Katerji et al. 1994, Akhtar et al. 2003). The latter seems crucial under salinity, as without a successful crop stand simple emergence of embryonic tissues will prove futile. The process of germination is influenced by the nature and extent of salinity and, above all, the behavior of seeds or propagules (Tavili and Biniiaz 2009, Wahid et al. 2009). To better understand the adverse effects of salinity, we have categorized the process of germination into four events: (1) imbibition, (2) active metabolism, (3) emergence and elongation of embryonic tissues, and (4) establishment of seedlings (Table 12.1).

### 12.2.1 IMBIBITION OF WATER

Hydration of stored materials is the initial step for the onset of germination (Katembe et al. 1998). The osmotic component of salinity has a strong inhibitory effect on hydration of the embryo, cotyledon/s, and endosperm (Wahid et al. 1998). It is independent of salinity type and growth media,

**TABLE 12.1**  
**Effect of Salinity Type on Seed and Propagule Germination Processes**

| Germination Event                             | Salinity Applied                                                               | Response Elicited                                                                                                                                                                                                                                          | References                                                                                                                                 |
|-----------------------------------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| Imbibition                                    | NaCl                                                                           | Reduced hydration of embryo and cotyledon                                                                                                                                                                                                                  | Poljakoff-Mayber et al. (1994) and Wahid et al. (1998)                                                                                     |
| Active metabolism                             | NaCl + Na <sub>2</sub> SO <sub>4</sub> + CaCl <sub>2</sub> + MgCl <sub>2</sub> | Reduced water uptake                                                                                                                                                                                                                                       | Ashraf et al. (1989)                                                                                                                       |
|                                               | NaCl                                                                           | Disaggregation of inter-membrane particles: leakage of solutes; reduced mobilization of reserves; inhibited activities of carbohydrates and fatty acid metabolism enzymes; altered patterns of protein synthesis; production of osmotically active solutes | Yupsanis (1994), Dell'Aquila and Spada (1993), Wahid et al. (1998), Small and Gutterman (1992), Rogers et al. (1995), and Ramagopal (1990) |
|                                               |                                                                                | Lower loss of cotyledon dry weight                                                                                                                                                                                                                         | Ruffino et al. (2009)                                                                                                                      |
|                                               |                                                                                | Ultrastructural changes in germinating embryos                                                                                                                                                                                                             | Petruzzelli et al. (1992)                                                                                                                  |
| Emergence and elongation of embryonic tissues | NaCl                                                                           | Delayed and reduced emergence of radicle and plumule; reduced elongation of embryonic tissues                                                                                                                                                              | Bliss et al. (1986), Rogers and Noble (1991), Stumpf et al. (1986), and Wahid et al. (1997)                                                |
|                                               | NaCl                                                                           | Inhibition of seed germination                                                                                                                                                                                                                             | Turhan and Ayaz (2004)                                                                                                                     |
|                                               | CaCl <sub>2</sub>                                                              | Reduced emergence of seedlings                                                                                                                                                                                                                             | Katerji et al. (1994)                                                                                                                      |
|                                               | NaCl + Na <sub>2</sub> SO <sub>4</sub> + CaCl <sub>2</sub> + MgCl <sub>2</sub> | Inhibited radicle emergence                                                                                                                                                                                                                                | Niazi et al. (1987)                                                                                                                        |
|                                               | NaCl + Na <sub>2</sub> SO <sub>4</sub> + MgCl <sub>2</sub> + CaSO <sub>4</sub> | Delayed emergence and final suppression of embryonic tissues growth                                                                                                                                                                                        | Sinha and Gupta (1982)                                                                                                                     |
|                                               | Seawater                                                                       | Delayed rate and emergence of seedlings                                                                                                                                                                                                                    | Villiers et al. (1994)                                                                                                                     |
|                                               | NaCl + Na <sub>2</sub> SO <sub>4</sub> + MgSO <sub>4</sub>                     | Inhibited growth of seedlings                                                                                                                                                                                                                              | Huang and Redman (1995)                                                                                                                    |
|                                               | NaCl + CaCl <sub>2</sub> + MgSO <sub>4</sub>                                   | Reduced growth of seedlings                                                                                                                                                                                                                                | Al-Mutawa (2003)                                                                                                                           |
| Establishment of seedlings                    | NaCl                                                                           | Enhanced seedling mortality; reduced seedling Growth                                                                                                                                                                                                       | Stumpf et al. (1986) and Rogers et al. (1995)                                                                                              |
|                                               | NaCl + Na <sub>2</sub> SO <sub>4</sub> + MgCl <sub>2</sub> + CaSO <sub>4</sub> | Reduced growth, vigor and establishment of seedlings                                                                                                                                                                                                       | Sinha and Gupta 1982                                                                                                                       |

as any salt will induce an osmotic effect (Alam et al. 2003, Tavili and Biniiaz 2009). One study found that seeds of a halophytic shrub (*Atriplex lentiformis*) and a glycophytic species (*Medicago sativa*) imbibed water from a saline substrate in a similar manner, suggesting that initial events of seed germination were similar in both species (Malcolm et al. 2003).

### 12.2.2 ACTIVE METABOLISM IN STORAGE TISSUES AND EMBRYO GROWTH

Upon exposure to saline solutions, ions are inevitably taken up by the seed, which are toxic to various physiological and biochemical processes. The activities of enzymes are hampered (Yupsanis et al. 1994, Yokoishi and Tanimoto 1994), leading to altered and reduced synthesis of micro- and macromolecules (Ben Miled-Daoud and Cherif 1994) and their reduced mobilization to developing

tissues (Wahid et al., 1998, Ben Miled-Daoud and Cherif 1994, Huang and Redman 1995). In *Pinus pinea* seeds, sodium chloride (NaCl) or seawater reduced the activity of isocitrate lyase, malate synthase, phosphoenolpyruvate carboxykinase, and citrate synthase, but NaCl was more damaging (Sidari et al. 2008). Applied salinity increased respiratory rate and ethylene production, enhanced the levels of spermidine and spermine, and reduced putrescine levels in germinating *Lactuca sativa* cultivars. These changes suggest that ethylene, but not polyamines, are a marker of salinity tolerance (Zapata et al. 2003, 2004).

Salinity, applied using NaCl and sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), terminated mobilization of storage lipids by day 8 in cotyledons and day 4 in roots of purple alfalfa seeds during germination and seedling growth (Baranova et al. 2006). Another study focusing on ultrastructural changes in alfalfa cotyledons revealed that an osmotic potential of 607.8 kPa developed using NaCl did not suppress transformation of amyloplasts into chloroplasts and utilization of starch, while  $\text{Na}_2\text{SO}_4$  inhibited development of chloroplasts and starch utilization. Thus, based on these cytological analyses, a model can be developed for the utilization of starch grains and formation of photosynthetic compartments of chloroplasts in the mesophyll of cotyledons (Baranova et al. 2007).

In cashew (*Anacardium occidentale*) nuts, applied salinity delayed seedling growth and mobilization of cotyledonary reserves. It also affected mobilization of proteins prior to breakdown of lipids and starch. Globulin was the predominant storage protein in cotyledons, and its mobilization and seedling establishment was delayed by NaCl. Free amino acids were mainly retained by cotyledons, while partitioning of storage proteins, lipids, and starch to embryonic tissues was delayed (Voigt et al. 2009). Dell'quila and Spada (1993) noted the synthesis of new proteins in response to saline stress in wheat embryos, which disappeared on return to water. This pattern of protein synthesis in alfalfa and metabolism of carbohydrates was attributed to the specific effect of ions on the activities of pertinent enzymes (Yupsanis et al. 1994, Promila and Kumar 2000). Salinity also improved accumulation of soluble sugars, free proline, and soluble proteins in various plant species (Poljakoff-Mayber et al. 1994, Zidan and Elewa 1995). These metabolites are beneficial for embryo growth by reducing osmotic inhibition and providing substrates for elongation of embryonic tissues (Lin and Kao 1996b, Wahid et al. 1998, Thakur and Sharma 2005).

### 12.2.3 EMERGENCE AND ELONGATION OF EMBRYONIC TISSUES

Seed reserves are used for embryo growth and elongation of young tissues, and involve the turnover and *de novo* synthesis of macromolecules. Germinating seeds in saline media exhibited a lowered and delayed production of radicle and plumule (Wijte and Gallagher 1996b, Wahid et al. 1997, Machado Neto et al. 2004). Sodium chloride affects the emergence of young tissues more adversely than other salinity types (Katerji et al. 1994, Wahid et al. 2009). Soybean seeds, subjected to germinate in NaCl solutions, displayed increased  $\text{Na}^+$  and reduced water,  $\text{Ca}^{2+}$  and  $\text{K}^+$  contents, which were the main reasons for reduced growth of embryonic tissues (Khajeh-Hosseini et al. 2002). However, ionic stress induced proline production in the seed, which played an important role in better seed germination and production of embryonic tissues in sorghum (Thakur and Sharma 2005).

### 12.2.4 SEEDLINGS ESTABLISHMENT

A successful crop stand depends on the establishment of young seedlings. Prolonged exposure to substrate salinity results in an extremely poor stand (Mondal et al. 1988) caused by seedling mortality (Wahid et al. 1999). This may be more pronounced in the case of glycophytes owing to their high sensitivity to salinity (Khan and Ungar 1997b). A good stand of *Sorghum halepense* under mild salinity was attributed to the rapid rate of germination (Sinha and Gupta 1982). Plants with more seedling vigor also produced better stands under saline conditions in tepary and navy beans (Goertz and Coons 1991). The vigorous growth of seedlings in relatively sensitive varieties of *Catharanthus roseus* was attributed to proline metabolism under salinity stress (Jaleel et al. 2007). Benmahioul et al. (2009) reported that in

*Pistacia vera*, salinity had no influence on the production of embryonic axes; rather, postgermination mortality was a major factor in seedling survival. In addition, seed germination tendency in euhalophyte *Suaeda salsa* determined final plant density and survival under salinity (Song et al. 2008).

Investigations on three forest coniferous species, *Picea mariana*, *P. glauca*, and *Pinus banksiana*, revealed that seedling emergence in *P. banksiana* was the lowest but *P. glauca* was the most salt sensitive. High concentrations of sodium sulfate caused hypertrophia in all the species, resulting in reduced seedling height, weight, root length, and number of roots in these species (Croser et al. 2001). In Argan (*Argania spinosa*) plants, reduced seedling survival and growth was attributed to ion toxicity as evidenced from symptoms of salt damage on seedlings (Bani-Aameur and Sippl-Michmerhuizen 2001).

## 12.3 SALINITY EFFECTS ON SEED GERMINATION

Salinity substantially reduces seed germination because of its osmotic effects; mineral toxicity also interferes with germination metabolism. The effects of salinity on seed germination are discussed below.

### 12.3.1 GERMINATION AND SALINITY: OSMOTIC AND TOXICITY EFFECTS?

Germination and salinity interaction is often studied on the premise that it has a dual action, that is, osmotic and toxic actions (Norlyne and Epstein 1984). Attempts to separate both components of salinity by using isotonic solutions of salts and nonpermeating osmotica yielded conflicting data (Petrizzelli et al. 1991, Corchete and Guerra 1986). Some regard the osmotic effect as the crippling factor (Rogers and Noble 1991, Debez et al. 2004), whereas most consider ion toxicity to be the noxious component (Gomes Filho and Sodek 1988, Yupsanis et al. 1994, Wahid et al. 1999, Lin and Kao 1996b), or that both components are equally detrimental to germination (Von der Moezel and Bell 1988, Song et al. 2005a, Sebei et al. 2007, Wahid et al. 2009). Wahid et al. (1998) reported that incubation of seeds in salt solution followed by reduced germination in water inferred ion toxicity. The nature and type of salinity may have varying effects on germination due to differences in ion activity. Most reports find more damaging effects of chloride than sulfate ions (Akhtar et al. 2003, Epstein and Bloom 2005, Vicente et al. 2007).

### 12.3.2 METABOLISM OF STORED MATERIALS

Seeds, whether monocots or dicots, contain storage tissues (endosperm and cotyledons, respectively) and an embryo. The nature and extent of stored materials may differ between species. Major stored materials include proteins, sugars, and oils, with smaller amounts of nucleic acids, plant growth regulators, nitrogenous compounds other than proteins, and some nutrients (Bewley and Black 1985). Salt stress hampers the metabolism of stored materials and embryo growth. At the onset of germination, synthesis of enzymes and changes in metabolic pattern are initiated (Khan and Ungar 1997a), but salinity either alters or does not permit synthesis of specific metabolites required for germination (Gomes Filho and Sodek 1988, Guerrier 1988, Yupsanis et al. 1994).

Salinity stress hampers utilization and mobilization of materials required for seedling production by affecting enzymatic activities of seed essential for these reactions (Table 12.2). For instance, wheat seeds treated with increasing NaCl concentrations or decreasing osmotic potential of germination media resulted in increased proline synthesis and decreased protein contents; whereas activities of proline metabolism enzymes initially increased but then decreased and became steady. These changes suggested that proline accumulation cannot be considered as an index of resistance for salinity stress (Song et al. 2005b). This group, in a separate study, reported that activation of the antioxidant system is an important component of salinity tolerance in wheat during germination (Lei et al. 2005).

**TABLE 12.2**  
**Metabolic Changes in Germinating Seeds and Propagules under Salinity Stress**

| Metabolic Activity | Effect of Salinity                                                                                         | References                     |
|--------------------|------------------------------------------------------------------------------------------------------------|--------------------------------|
| Proteins           | Toxic to the protein phosphatase and protein kinase specific activities                                    | Guerrier (1988)                |
|                    | Inhibition of methionine and leucine uptake and incorporation into proteins chain                          | Dell'Aquila and Spada (1993)   |
|                    | <i>De novo</i> synthesis of four new heat-shock proteins during stress and seven during recovery           | Ramagopal (1990)               |
|                    | Changes in protein phosphorylation-dephosphorylation                                                       | Yupsanis et al. (1994)         |
| Carbohydrates      | Reduction in endospermic $\alpha$ -amylase activity in a concentration-dependent manner                    | Ashraf et al. (2002)           |
|                    | Promotion of cotyledonary $\alpha$ -glycosidase activity                                                   | Corchete and Guerra (1986)     |
|                    | Accumulation of osmotically active sugars                                                                  | Zidan and Elewa (1995)         |
|                    | Increase in the levels of Total soluble sugars, sucrose and glucose concentrations in the cotyledons       | Ruffino et al. (2009)          |
| Nucleic acids      | Inhibition of RNase activity                                                                               | Gomes Filho and Sodek (1988)   |
|                    | Hydrolysis of RNase during germination                                                                     | Gomes Filho et al. (2008)      |
|                    | Decrease in DNA contents during germination                                                                | Gomes Filho et al. (1983)      |
|                    | Reduced incorporation of the precursors of nucleic acids biosynthesis                                      | Petruzzelli et al. (1991)      |
| Lipids             | Reduction in activity of glyoxysomal enzymes; reduction in utilization of total lipids and triacylglycerol | Miled-Daoud and Cherif (1994)  |
|                    | Increase in linolenic acid and linolenic acid-rich TAGS                                                    | Sebei et al. (2007)            |
| Other compounds    | Increase in betaine aldehyde dehydrogenase enzyme activity                                                 | Yokoishi and Tanimoto (1994)   |
|                    | Increased betaine contents                                                                                 | Poljakoff-Mayber et al. (1994) |
|                    | Progressive accumulation of free proline                                                                   | Wahid et al. (1998)            |
|                    | Decrease in soluble amino nitrogen                                                                         | Yokoishi and Tanimoto (1994)   |
| Seed minerals      | Reduced levels of macro- and micronutrients in seed                                                        | Koyro and Eisa (2008)          |
|                    | Reduced levels of macro- and micronutrients in bud                                                         | Wahid et al. (2009)            |

### 12.3.2.1 Proteins

Salinity impacts enzyme activity via protein metabolism (Yupsanis et al. 1994, Small and Gutterman 1992). Applied salinity increased protein concentrations in bean (*Phaseolus vulgaris*) cultivars during germination (Dantas et al. 2007). Activity of proteases, which catalyze the turnover and solubilization of proteins to soluble nitrogen in seed, are largely prone to salinity (Bose et al. 1982). Salinity interfered with the incorporation of (<sup>3</sup>H)leucine and (<sup>35</sup>S)methionine during protein synthesis in wheat embryos (Dell'Aquila and Spada 1993, Petruzzelli et al. 1991) and modulated production of a selected group of proteins not synthesized otherwise (Dell'Aquila and Spada 1993). Ramagopal (1990) identified eight new proteins in germinating barley embryos under salt stress and seven during recovery. In two flax (*Linum usitatissimum*) cultivars, soluble protein contents reduced with time, while salt-induced precocity of protein degradation occurred at 200 mM NaCl, reducing availability of proteins to embryonic tissues (Sebei et al. 2007).

### 12.3.2.2 Carbohydrates

Carbohydrates (as starch) are a major component of storage material in some seeds (e.g., caryopses). Amylases mainly regulate carbohydrate metabolism, and their activity is greatly affected by salinity. The activity of  $\alpha$ -amylase is reduced under salinity in a concentration-dependent manner, depressing seedling growth (Lin and Kao 1996a). Greater salt tolerance of sorghum during germination was attributed to enhanced activity of  $\alpha$ -amylase (Promila and Kumar 2000). Salt-treated lentil seeds did not vary in different solute contents; however, activity of  $\alpha$ -galactosidase increased and caused accumulation of sucrose, galactose, and mannose in embryonic tissues (Corchete and Guerra 1986). An accumulation of osmotically active sugars and proline was noted in different plants, which played an important role during and after relief from salinity (Zidan and Elewa 1995, Wahid et al. 1998).

### 12.3.2.3 Lipids

Glyoxysomal enzymes are responsible for metabolism of stored lipids. Salinity exerts an inhibitory effect on glyoxysomal catalase, malate synthase, and iso-citrate lyase, decreasing levels of triacylglycerol, diacylglycerol, and monoacylglycerol, and increasing levels of free fatty acids and polar lipids (Ben Miled-Daoud and Cherif 1994). In two flax cultivars, applied salinity increased linolenic acid contents and linolenic acid-rich molecular species of triacylglycerols (TAGS) and decreased seed germination (Sebei et al. 2007).

### 12.3.2.4 Nucleic Acids

The most important factor in nucleic acid metabolism is changes in the activation and levels of ribonuclease (RNase). Salinity delays *de novo* synthesis and/or activation of RNase in *Vigna* cotyledons due to its toxic effect (Gomes Filho and Sodek 1988). RNase in the cotyledon of cowpea (*Vigna unguiculata*) under salinity stress hydrolyzed and played a protective role in seedling establishment (Gomes Filho et al. 2008). Cotyledonary RNA levels increased slightly during the first day of germination, but subsequently decreased for up to 7 days; however, DNA decreased continuously during this period (Gomes Filho et al. 1983). Petruzzelli et al. (1991) suggested that suppression of nucleic acid biosynthesis in wheat embryos was due to salt-induced inhibition of the incorporation of precursors into nucleic acids.

### 12.3.2.5 Other Organic Compounds

Various endogenous compounds are differentially metabolized during germination and seedling growth. The glycinebetaine, a compatible solute, disappears (Poljakoff-Mayber et al. 1994), exhibits no change (Stumpf et al. 1986), or accumulates as a result of salt-stimulated betaine aldehyde dehydrogenase activity and rescues the seed from the adverse effects of salt (Yokoishi and Tanimoto 1994). Similarly, increased free proline and soluble sugars of seeds or seedlings has a beneficial role (Poljakoff-Mayber et al. 1994, Wahid et al. 1998, Zidan and Elewa 1995, Wahid et al. 2007).

### 12.3.2.6 Seed Minerals

High contents of seed nutrients are vital for germination, but salinity suppresses their role in seed metabolism and seedling production (Gallasch and Dalton 1989). During germination of sorghum caryopses, more potassium (K), calcium (Ca), phosphorus (P), and nitrogen (N) was partitioned into the plumule and radicle as a strategy to tolerate salinity (Wahid et al. 1998). Guerrier (1986) attributed reduced salt tolerance in tomato to its inability to accumulate and transport lower amounts of Ca and K.

## 12.3.3 REPROGRAMMING OF GENE EXPRESSION

Salinity stress tolerance is a multigenic trait and involves the up- and downregulation of many genes (Taiz and Zeiger 2006); although studies reporting patterns of gene expression at seed germination are scarce. In *Amaranthus hypochondriacus*, a cDNA encoding a cysteine protease inhibitor

(*AhCPI*) synthesized a polypeptide of 247 amino acids with a putative N-terminal signal peptide, which had significant homology to other plant cystatins. *AhCPI* gene was constitutively expressed in mature seeds. Although *AhCPI* expression decreased in germinating seeds, it was active in the radicles and hypocotyls of seedlings. The encoded protein regulated seed germination and protected against various abiotic stresses, including salinity, by inducing accumulation of serine-proteinase-type protease inhibitors (Valdes-Rodriguez et al. 2007). Activation of protease inhibitors enhances salt tolerance during germination (Shan et al. 2008). In transgenic Arabidopsis, overexpression of *Zea mays* 12-oxo-phytodienoic acid reductases (*ZmOPRI*) conferred tolerance against high salt stress during germination in roots but hardly in leaves (Gu et al. 2008).

## 12.4 GERMINATION OF PROPAGULES UNDER SALINITY

The initial events of propagule germination may differ from seeds since propagules are vegetative and seeds are reproductive. However, bud sprouting, elongation, and seedling establishment appear similar. The rate and percentage of germination of sugarcane nodal buds (stem cuttings) significantly decreased in response to NaCl damage (Wahid et al. 1997, Akhtar et al. 2003). These plants had more Na<sup>+</sup> and Cl<sup>-</sup>, and a concomitant reduction in potassium, calcium, nitrogen and phosphorus, reduced elongation, and less seedling dry matter. Histological studies revealed that sprouting buds depicted an array of changes during transition from undifferentiated until the complete differentiation of mesophyll and vascular tissues under salinity stress (Rasheed 2009). In a recent study, Wahid et al. (2009) found a positive correlation between essential nutrient contents in nodal buds of sugarcane genotypes and salinity tolerance at sprouting and during seedling growth.

There is a dearth of information about salt tolerance of propagules during germination. Citrus rootstocks used to raise plantlets under salinity had a negative correlation of Cl with certain endogenous nutrients (Gallasch and Dalton 1989). Resting buds of salt-stressed poplar plants, grown in vitro, did not accumulate glycinebetaine and proline, reducing seedling growth (Bray et al. 1991). Similarly, tubers of hydrilla had signs of salt damage and reduced germination (Stewart and Van 1987). In some potato species and cultivars, studies involving micropropagation of axillary buds in the presence of NaCl significantly reduced shoot length and fresh weight of sprouts (Donnelly et al. 2007).

## 12.5 REGULATION OF IONS IN SEEDS AND SEEDLINGS

Exposure of seeds or seedlings to salinity results in an influx of ions with the imbibition of water, which has an adverse effect on embryo growth (Poljakoff-Mayber et al. 1994). This may lead to a marked decrease in the internal potassium concentration (Petruzzielli et al. 1991, Nichols et al. 2009), a vital nutrient for protein synthesis and plant growth (Epstein and Bloom 2005). Seedlings exposed to salinity are highly prone to excessive ions, sometimes leading to their death shortly after emergence (Wahid et al. 1999, Ahmad et al. 2005). The ability of plants to cope with ion toxicity is principally related to the greater transport of ions to shoots. Grasses tolerate salt by storing toxic ions in the mesocotyl up to a certain limit (Drew and Lauchli 1987, Wahid et al. 1998). As a result, the epicotyls and hypocotyls avoided ion toxicity, thus ensuring better growth (Wahid et al. 1998, Fardus 2006).

## 12.6 STRUCTURAL CHANGES IN SEEDS AND SEEDLINGS UNDER SALINITY

Salinity triggers structural changes at various levels of organization in seeds and seedlings (Table 12.3). At the subcellular level, major changes were found in (1) nuclear chromatin, which was condensed suggesting suppressed nucleic acid biosynthesis, (2) formation of small provacuoles instead of a single large vacuole, and (3) damaged mitochondrial apparatus and reduced oxygen uptake (Bliss et al. 1986, Petruzzielli et al. 1991). Salinity caused contraction of the plasmalemma away from cell walls (Petruzzielli et al. 1991) due to disaggregation of intramembranous particles (Bliss et al. 1984). The cell wall of salt-treated cotton roots and sorghum mesocotyl thickened considerably (Reinhardt and Rost 1995, Wahid et al. 1998).



**TABLE 12.3**  
**Salt-Induced Changes in Anatomical Characteristics in Seeds and Embryonic Structures during Germination**

| Level of Organization | Salt-Induced Change                                                     | References                |
|-----------------------|-------------------------------------------------------------------------|---------------------------|
| Subcellular           | Formation of small provacuoles in coleorhiza cells                      | Bliss et al. (1986)       |
|                       | Diffusion and condensation of chromatin material in embryo              | Petruzzelli et al. (1991) |
|                       | Reduced size of plasmalemma and mitochondria                            | Petruzzelli et al. (1991) |
|                       | Lignification and thickening of cell wall                               | Wahid et al. (1998)       |
| Cellular              | Reduced size of cortical cells in mesocotyl of sorghum                  | Wahid et al. (1998)       |
|                       | Induced exodermis and accelerated endodermal development                | Reinhardt and Rost (1995) |
|                       | Earlier development and differentiation of secondary xylem in hypocotyl | Reinhardt and Rost (1995) |
| Tissue                | Constriction of cortical tissue of mesocotyl                            | Wahid et al. (1998)       |
|                       | Increased lignification of secondary tissues                            | Valenti et al. (1992)     |
|                       | Reduction in size of perisperm in embryo                                | Koyro and Eisa (2008)     |

At cell and tissue levels, salinity stress reduced the cortical cell area and, as a result, the mesocotyl of sorghum was considerably constricted and appeared to act as a repository for ions (Wahid et al. 1998). In cotton, an exodermis with a casparian band containing suberin lamellae closer to the root base and in the transition zone of the hypocotyl was induced (Reinhardt and Rost 1995). This protected the loss of water or solute leakage from roots, which was important for osmotic adjustment. Salinity also stimulated development and lignification of secondary tissues and enhanced the number of water-storage cells in the epidermis and cortical layer of the hypocotyl (Valenti et al. 1992).

## 12.7 FACTORS INTERACTING WITH SALINITY DURING GERMINATION

There are various internal (plant) and external (environmental) factors that affect seed germinability under saline conditions, which are discussed below.

### 12.7.1 PLANT FACTORS

The most important plant factors affecting germination under saline conditions are the seed coat, dormancy, seed vigor, and age.

#### 12.7.1.1 Seed Coat and Seed Coverings

The seed coat is the first barrier to the entry of water and ions into the seed. The hard and thick seed coat causes a state of dormancy and restricts entry of water into the seed, minimizing contact of ions with the embryo (Taiz and Zeiger 2006). It also acts as a buffering agent to ionic toxicity (Poljakoff-Mayber et al. 1994, Eschie 1995) and enhances germination. The role of seed coverings exposed to salinity at germination depends upon the plant species under investigation. In cases where the seed coat offers no barrier to the movement of ions and water, it may accumulate toxic ions, minimizing their access to the embryo. For example, seeds of *Acacia tortilis* and *A. coriacea* accumulated more Na<sup>+</sup> in the seed coat and leaked less K and Ca from the embryo (Rehman et al. 1998).

In contrast, the hull on the achene of sunflower played no role in salinity tolerance during germination (Kaya 2009). In *A. portulacoides*, the presence of bracteoles inhibited germination, particularly with increasing salinity. The bracteoles appeared to induce some sort of dormancy, thus crippling germination (Redondo-Gomez et al. 2007).

#### 12.7.1.2 Dormancy

One of the primary impacts of salinity is the induction of dormancy in the seed either from inhibition of nucleic acid synthesis (Khan 1971) or plant growth regulator imbalance (Khan and Rizvi 1994). Although dormancy has no consistent relationship with salinity (Cruz et al. 1995), it is important for halophytes since it permits the seed to remain viable until the environment becomes conducive to germination (Fenner 1985, Khan and Ungar 1997a). Wetson et al. (2008) reported that the state of dormancy induced by salinity and low seed temperature of *Suaeda maritime* was released when the temperature increased to about 15°C and salinity was also reduced.

#### 12.7.1.3 Seed Age

Aging or prolonged storage of seeds affects their germinability (Schoettle and Leopold 1984). This has been used to test and predict salt-tolerance potential during germination. In alfalfa, a strong negative relationship existed between salt tolerance and seed age in a sensitive genotype; but there was a significant decline in solute leachate during imbibition of water in a tolerant genotype (Smith and Dobrenz 1979). In soybean, seed vigor was a major factor in the recovery of aged seeds during germination from salinity stress (Khajeh-Hosseini et al. 2003).

#### 12.7.1.4 Seed Polymorphism

Seed polymorphism, in terms of color, size or mass, has adaptive value in the germination and subsequent life cycle of numerous plants species especially halophytes (Khan and Weber 2008). For instance, seed size of two halophytes, *Salicornia europaea* and *A. triangularis*, was proportional to salt tolerance. In the same saline medium for germination, small seeds had more toxic ions and less reserves per unit weight, which led to more dormancy-delayed germination and reduced weight of seedlings than larger seeds (Khan and Ungar 1984a,b, Ungar 1987). Likewise, in the genus *Frankia*, larger seeded species germinated better under salinity than those with smaller seeds (Easton and Kleindorfer 2009). In contrast, small seeds of the glycophytic sunflower germinated and grew more rapidly in saline conditions than large seeds (Kaya and Day 2008).

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In addition to seed size, seed color has an adaptive value in salt tolerance. In *Suaeda splendens*, brown-colored seeds germinated and survived in a highly saline environment better than black-colored seeds (Redondo-Gomez et al. 2008). Likewise, dark-green-colored seeds of pea germinated better in saline conditions than light-green-colored ones (Atak et al. 2008).

#### 12.7.1.5 Seed and Seedling Growth and Vigor

Seed vigor incorporates properties of the seed that determine the performance of the seed or a seed lot during germination and seedling emergence (Sun et al. 2007). Healthy and vigorous seeds display better germination and seedling growth, which is important for the establishment of plants in the field. As for seedling vigor, root growth, being the most important factor, determines the

establishment of a stand under salinity (Lin and Kao 1996a, Coons et al. 1990). This problem may be partially solved either by using a higher seed rate to obtain high seedling density or by selecting crops for high seedling vigor, especially in arable farming (Rogers and Noble 1991).

### 12.7.2 ENVIRONMENTAL FACTORS

Environmental factors influencing germination include temperature, light, water, and gases.

#### 12.7.2.1 Temperature

A slight variation in temperature can have a major impact on germination. The adverse effect of high salinity is further aggravated by higher temperature regimes, which prolonged seedling emergence in some halophytic plants (Khan and Ungar 1996, 1997a, El-Keblawy and Al-Rawai 2005) or completely inhibited seed germination in the xerohalophyte *Reaumuria vermiculata* (Gorai and Neffati 2007). However, moderate temperatures were more favorable to germination in *Panicum turgidum* (Al-Khateeb 2006). Seed germination of *Limonium stocksii*, exposed to 200 mM NaCl or more, was not affected at moderate (20°C–30°C) temperatures, but was inhibited at lower (10°C–20°C) or higher (25°C–35°C) temperatures (Zia and Khan 2004). Likewise, germination percentage in three euhalophytic species severely declined at the lowest (10°C) and highest (35°C) temperatures under 500 mM level of NaCl (Song et al. 2006).

#### 12.7.2.2 Light

Light has a profound effect on the germination of many species (Agami 1986). Light may be effective in breaking dormancy and promoting germination in some halophyte species. For instance, seeds kept in complete darkness germinated poorly under salinity than those kept in 12 h light/dark periods in *Aeluropus lagopoides*, *Halopyrum mucronatum*, *Sporobolus ioclados*, and *Urochondra setulosa* (Khan and Gulzar 2003), and *L. stocksii* (Zia and Khan 2004). This may result in better stand establishment in marginally saline areas (De Villiers 1994, Khan and Ungar 1997a).

#### 12.7.2.3 Water

Applied salinity is intricately linked to water availability and seed germination. Salt-induced lowering of water potential in the germination medium enhances toxicity, whereas scarcity of water further aggravates it (Atak et al. 2006). Reduced moisture contents in germination media coupled with high ionic strength leads to a differential pattern of protein synthesis (Petruzzelli et al. 1991, Dantas et al. 2007), delayed or inhibited emergence of embryonic tissues (Sinha and Gupta 1982, Yildirim and Güvenç 2006), and a decrease in final rate and percentage, and index of germination (Miyamoto et al. 1982, Mahdavi and Sanavy 2007). Incidental availability of water to salinity-stressed seed can improve seed germination until there is no permanent damage to embryo. This happens because the ionic toxicity is minimized (Redman 1974, Badger and Ungar 1989, Abbassi and Koocheki 2008).

#### 12.7.2.4 Gases

Availability of gases including oxygen (O<sub>2</sub>), carbon monoxide (CO), and nitric oxide (NO) can affect seed germination under salinity. Salt-induced dormancy reduces the availability of oxygen to the embryo for metabolic activities. High salinity coupled with hypoxia significantly reduces both emergence and elongation of the radicle and plumule (Wijte and Gallagher 1996a). Anoxia completely restricted the process of germination; however, no specific difference was discernible with respect to salinity and anoxia tolerance. *Spartina alterniflora* better tolerated salinity and anoxia than *Phragmites australis*, due to its more rapid rate of coleoptile and mesocotyl growth (Wijte and Gallagher 1996b). Use of increased concentrations of hematin as CO donor improved wheat seed germination under salinity by improving antioxidant activities and reducing lipid peroxidation and degradation of seed reserves (Xu et al. 2006). Likewise, soaking wheat seeds in 0.1 mM of sodium nitroprusside (SNP; as NO donor) for 20 h improved seed germination at

300 mM NaCl (Zheng et al. 2009). This action of NO in improving seedling emergence in *Suaeda salsa* was due to signaling property of NO in activating seed-dormancy-breaking genes induced by salinity stress (Song et al. 2009).

## 12.8 CONCLUSION

The events of germination, from imbibition of water to seedling establishment, are all adversely affected by increased levels of salinity. Salinity hampers the rate and percentage of germination, partially through the osmotic effect on imbibition of water and is mainly due to its toxicity to the metabolism of seed reserves. Endogenous nutrient concentrations in seeds and propagules are important for their salinity tolerance. Salinity also induces structural changes at subcellular, cellular, tissue, and organ levels and affects the rate of respiration, transport of materials, and induction of new tissues in seeds or seedlings. As a multigenic trait, salinity alters the expression of a number of genes which are helpful in improving salinity tolerance in germinating seeds. Changes in enzymatic activities may be another important factor in modulating seed germination.

Certain internal and external factors substantially interact with germination and seedling growth under salinity. The seed coat minimizes access of ions to the embryo. Dormancy allows halophytes to escape the adverse effect of salinity. Aging has been used to test seed viability and to predict salt-tolerance ability. Larger seeds tend to have better germination and seedling vigor because of a higher content of seed reserves and lower absorption of toxic ions per unit weight. Likewise, seed color has been found to be related to salinity tolerance in a few plant species. Hard and tough seed coats and seed coverings inhibit seed germination. Salinity-induced dormancy creates an anoxic condition and inhibits seed germination. Scarcity of water, prevailing higher temperatures, and absence of light, all affect seed germination and production of embryonic structures. The seeds with high vigor produce stout seedlings, which are better able to withstand saline conditions and produce better stands in the field.

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# 13 Response of Crop Plants to Nitrogen Stress: Opportunities to Increase Nitrogen Use Efficiency

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### 13.1 INTRODUCTION

People on earth are alive because functional and structural proteins in their bodies are made of nitrogen (N) derived from plant or animal food. A great proportion of this N has its origin in fertilizers used in modern agriculture that evolved significantly during the green revolution. The invention of the N fertilizer was possible due to the greatest scientific contribution of Fritz Haber, who discovered the method to convert nonreactive atmospheric nitrogen to reactive N in the form of ammonia (Smil 1997). This process has now significantly influenced the global N balance and has placed agroecosystems in a precarious condition, as fertilizer N is significantly contributing to pollution of aquifers, accumulation of greenhouse gases (Stern Review 2006, Schlesinger 2009), and depletion of the atmospheric ozone layer (Majumdar 2005). In addition, recent trends in the cost of fertilizers have led to a rise in the cost of food production.

Much of the concerns about N fertilizers emanate from the fact that only a fraction of applied N is utilized by crop plants. Persistent research efforts for reduced use of reactive N in agriculture have led to innovative agronomic practices that comprise slow-releasing N fertilizers and precision farming (Cassman et al. 2002). Organic farming is being increasingly proposed as another option to protect the environment. However, if all farmers adopt organic farming, the food produced will not be sufficient to feed the global population, as natural N<sub>2</sub> fixation and organic N recycling cannot keep pace with the N demand of high-yielding crop cultivars (Smil 1997). On the other hand, recent studies with simulation modeling reveal that the N balance tends to be negative in areas such as Indo-Gangetic plains that are being subjected to intensive agriculture mostly with rice and wheat rotation (Pathak et al. 2006). Hence, it is predicted that the next few generations of humankind will continue to depend on reactive N generated in fertilizer factories (Smil 1997). Thus, there is an urgent need for the development of a comprehensive approach to optimize reactive N utilization in every sphere of life. This can be accomplished to a considerable extent by managing the N fertilizer (Ju et al. 2009) and improving the nitrogen use efficiency (NUE) of crop plants through a combination of genetic improvement and agronomic approaches (Delmer 2005, Doberman 2006, Hirel et al. 2007).

The challenges associated with N and the need for improved nutrient efficiency have been emphasized in a few recent reviews (Pathak et al. 2008, Garnett et al. 2009, Townsend and Palm 2009). In an earlier review on NUE, Cassman et al. (2002) addressed the global challenge of meeting increased food demand and protecting environmental quality in cropping systems that produce maize, rice, and wheat. Quantitative understanding of current levels of N use, efficiency and losses in these systems, the biophysical controls on these factors, and the economic returns from adoption of improved management practices were highlighted in this review for setting the research agenda and developing effective policies. Pathak et al. (2008) have summarized the physiological, biochemical, and molecular aspects of NUE, quantitative trait loci (QTL) mapping studies, as well as transgenic efforts to improve NUE in crops and model plants. They also listed the transgenes used for improving NUE and its components. Garnett et al. (2009) elaborated root-based approaches for improving NUE by focusing exclusively on N uptake mechanisms at the root level, and also discussed the scope for molecular intervention.

The objective of this chapter is to present an overview of the present status of N utilization in agriculture, NUE including the process and magnitude of loss of applied N, progress in improving NUE, and strategies and opportunities for genetic improvement of NUE in crop plants.

### 13.2 PRESENT STATUS OF N FERTILIZER USE

The global N consumption has now crossed 100 million tons (Mt) (Heffer and Prud'homme 2008), with more than 50% of it being consumed by Asian countries (FAO 2008). It is estimated that 53.0Mt of N was applied to cereals in 2006–2006/2007, representing 55.3% of global fertilizer N consumption (Heffer 2009). Wheat and maize both contribute to 17.3% of the global use, followed

by rice (15.8%). Other cereals receive 4.6% of the fertilizer N. Oil crops are minor consumers of the N fertilizer, with a market share of 6.3% (6.0Mt of N), rapeseed/canola being the main consumer. Cotton and sugar crops represent 3.8% and 3.3% of the global fertilizer N consumption, respectively. Fruits and vegetables account for 15.3% of the total, and other crops account for 15.9% (Heffer 2009).

It is expected that fertilizer consumption in Africa and Latin America will increase substantially in the future. However, crops in a large proportion of land in these regions will remain undernourished. Hence, increased productivity with less application of the N fertilizer in general and enhanced productivity with no or marginal application of N in low-N-consuming regions are inevitable options for increased production in the future. Some of the fertilizer N applied in one crop season is retained in the organic matter of the soil and will be available in the subsequent crop season (Westerman and Kurtz 1972).

### 13.3 NITROGEN USE EFFICIENCY

#### 13.3.1 DEFINITION

NUE is measured in various ways, for example, the average yield produced per unit of N applied, the quantity of extra N contained in the crop per unit of N applied, or the extra yield produced per unit of N taken up by the crop (Buresh et al. 2007, Sylvester-Bradley and Kindred 2009). All of these ratios can be used as measures of the efficiency with which N is used. NUE is a term used to indicate the relative balance between the amount of fertilizer N taken up and used by the crop versus the amount of fertilizer N “lost.”

#### 13.3.2 PRESENT STATUS OF NUE IN CROP PLANTS

Achieving synchrony between N supply and crop demand is the key to optimizing trade-offs among yield, profit, and environmental protection in both large-scale systems in developed countries and small-scale systems in developing countries (Cassman et al. 2002).

Setting the research agenda to synchronize N supply and crop demand without excess or deficiency requires a quantitative understanding of current levels of NUE and losses in these systems. Based on global consumption and N removed by grains, Raun and Jounson (1999) estimated that the world cereal grain NUE is about 33%; however, a wide range of NUE values have been reported for different crops under different agroecological environments (Table 13.1).

#### 13.3.3 MECHANISMS OF LOSS OF FERTILIZER N

The N supply is determined not only by the applied fertilizer but also influenced largely by the type of soil, the microenvironment of the rhizosphere, and various mechanisms by which the applied fertilizer is lost into the environment. On the other hand, crop demand is determined by the genotype and its growth environment, for example, the levels of radiation and soil moisture. The two predominant N loss mechanisms that commonly occur are leaching and denitrification of the nitrate-N. Nitrogen loss due to the volatilization of surface-applied urea-based products is a third source of N loss for some fields. Surface runoff, particularly during heavy precipitation immediately after fertilizer application, can also lead to significant N loss. It has also been shown that substantial N is lost in the form of  $\text{NH}_3$  from aerial parts of crop plants. Major mechanisms of loss of N are described in brief in Sections 13.3.3.1 through 13.3.3.6.

##### 13.3.3.1 Leaching of Nitrate-N

All applied N fertilizer sources eventually convert completely into the nitrate-N form. This form of N is not held tightly by soil particles and can be leached from the soil profile with excessive rains,

**TABLE 13.1**  
**Values of NUE for Different Field Crops and Forage Grasses**

| Crop                                                                          | NUE       | Unit                                                          | Soil Type                                                                                                                                            | Location                                                                        | Crop Season | References                            |
|-------------------------------------------------------------------------------|-----------|---------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-------------|---------------------------------------|
| Rice, 13 genotypes (irrigated-lowland rice conditions, from a specific cross) | 56.6–63.9 | kg grain kg <sup>-1</sup> N in leaves at heading (UTE)        | An Entic Pelludert (fine, montmorillonitic, and thermic), with a sand, silt, and clay composition of 3.2%, 32.4%, and 64.4%, respectively; pH at 6.0 | Beaumont (29°57' N lat; 94°30' W long), Texas                                   | Summer      | Samonte et al. (2006)                 |
| Rice, 28 cultivars (110, 45, and 57 kg N ha <sup>-1</sup> )                   | 44–52     | kg grain kg <sup>-1</sup> N applied                           | —                                                                                                                                                    | Kyonggido Agricultural Research and Extension Services, Korea                   | —           | Lee et al. (2004)                     |
| Rice, 4 cultivars                                                             | 14.3–21.8 | kg grain (N Plot-0NPlot) kg <sup>-1</sup> N applied in N Plot | Typic Ustipsamment (Fatehpur loamy sand)                                                                                                             | Ludhiana, India                                                                 | Wet season  | Singh et al. (2002)                   |
| Rice, 3–5 high-yielding varieties                                             | 59–64     | kg grain kg <sup>-1</sup> N in leaves at heading (UTE)        | —                                                                                                                                                    | IRRI (tropical site), and at Taoyuan Township, Yunnan, China (subtropical site) | —           | Ying et al. (1998)                    |
| Rice, 180 genotypes (without N, 2 year experiment)                            | 61.9–82.7 | Average increase in GY for each kilogram increase in N uptake | —                                                                                                                                                    | IRRI, Philippines                                                               | Dry season  | Tirol-Padre et al. (1996)             |
| Barley, 25 genotypes (different locations)                                    | 35–48     | kg grain kg <sup>-1</sup> N                                   | —                                                                                                                                                    | Alberta, Canada                                                                 | —           | Anbessa et al. (2009)                 |
| Barley                                                                        | 31–50     | kg grain (N Plot-0NPlot) kg <sup>-1</sup> N applied in N Plot | Typic Argiudoll and a Petrocalcic Paleudoll                                                                                                          | Balcarce (37°45'S, 58°18'W), Argentina                                          | Wet season  | Computed based on Sainz et al. (2004) |

**TABLE 13.1 (continued)**  
**Values of NUE for Different Field Crops and Forage Grasses**

| Crop                                                                                 | NUE          | Unit                                                                    | Soil Type                                                                | Location                                                             | Crop Season   | References                            |
|--------------------------------------------------------------------------------------|--------------|-------------------------------------------------------------------------|--------------------------------------------------------------------------|----------------------------------------------------------------------|---------------|---------------------------------------|
| Barley                                                                               | 32.8         | kg grain kg <sup>-1</sup> N applied                                     | Coarse, sandy, clay-mixed montmorillonitic Typic Caliciorthid            | Obregon, Mexico (27°N 109°W, 40 masl)                                | Winter        | Ortiz-Monasterio et al. (1997)        |
| Maize (no-till irrigated, 0, 34, 67, 101, 134, and 202 kg N ha <sup>-1</sup> )       | 39–126       | kg grain kg <sup>-1</sup> available N (GY as a function of available N) | Fort Collins clay loam soil (fine loamy, mixed mesic Aridic Haplustalfs) | Fort Collins, Colorado                                               | Spring season | Halvorson and Curtis, (2007)          |
| Maize                                                                                | 50           | kg grain kg <sup>-1</sup> N uptake                                      | Reddish-brown clay soil (nitosol) at Harare and eutric fluvisol          | Harare (31°1'S, 17°49'E, and 1478 masl), Zimbabwe, and at Kenya      | Rainy season  | Mosisa et al. (2007)                  |
| Maize, 4 varieties, (no-till irrigated, 2 years, 70, 40, 210 kg N ha <sup>-1</sup> ) | 21.3 to 42.5 | kg GY (N Plot-0NPlot) kg <sup>-1</sup> N applied in N Plot              | Typic Argiudoll and a Petrocalcic Paleudoll                              | Balcarce (37°45'S, 58°18'W), Argentina                               | Wet season    | Computed based on Sainz et al. (2004) |
| Wheat                                                                                | 29.4         | kg grain kg <sup>-1</sup> N                                             | Fine Typic Cryaquept                                                     | MTT Agrifood Research Finland, Jokioinen, Finland (60°49'N, 23°30'E) | —             | Muurinen et al. (2006)                |
| Wheat (32, 43, 59, and 89 kg N ha <sup>-1</sup> )                                    | 34(10–70)    | kg grain kg <sup>-1</sup> N applied                                     | Coarse, sandy, clay-mixed montmorillonitic Typic Caliciorthid            | Obregon, Mexico (27°N 109°W, 40 masl)                                | Winter        | Ortiz-Monasterio et al. (1997)        |
| Oat                                                                                  | 27.1         | kg GY kg <sup>-1</sup> N applied                                        | Coarse, sandy, clay-mixed montmorillonitic Typic Caliciorthid            | Obregon, Mexico (27°N 109°W, 40 masl)                                | Winter        | Ortiz-Monasterio et al. (1997)        |

especially on lighter-textured soils. Nitrate-containing fertilizers, including liquid urea-ammonium-nitrate (UAN) solutions and ammonium nitrate, are susceptible to leaching loss as soon as they are applied. Urea can be converted to nitrate-N in less than two weeks in late spring, and, thereafter, is susceptible to leaching loss. Anhydrous ammonia is converted more slowly to nitrate-N because of its initial toxic effects on the soil microbes that are responsible for the conversion of ammonium-N

to nitrate-N. Loss of N due to leaching alone may be as high as  $36 \text{ kg ha}^{-1} \text{ yr}^{-1}$  in hillside agriculture with a slope of 7% (Zhu et al. 2009).

#### 13.3.3.2 Denitrification of Nitrate-N

Certain soil bacteria that thrive in saturated (anaerobic) soil conditions convert nitrate-N to oxygen and N gases. The volatilization of N gas can result in N losses of as much as 5% of the available nitrate-N per day (Hoeft 2004). Soils at greatest risk to denitrification are those that are naturally heavy and poorly drained, plus fields with significant levels of soil compaction that restricts natural drainage. Because denitrification affects nitrate-N, the relative risk of N fertilizer products is identical to that for loss of N due to leaching.

#### 13.3.3.3 Volatilization of Urea-Based Products

Urea-based N fertilizer products are susceptible to volatilization losses of N if surface-applied and not incorporated. Urease enzymes in the soil and plant residues convert the urea component to free ammonia gas. If this conversion occurs at the soil surface and is accompanied by warm sunny days, as much as 15%–20% of the urea-based N may volatilize within a week after application (Bundy 2001). If a half inch or more of rain occurs within the first 24 h after surface application, the risk of subsequent volatilization also drops to essentially zero (Bundy 2001). If the urea-based product is injected or mechanically incorporated after application, the risk of volatilization is essentially zero. Forms of N fertilizer susceptible to volatilization losses include dry urea or liquid UAN solutions that are surface-applied without incorporation. The risk of volatilization loss is greatest with high-residue cropping systems, warm sunny days after application, and surface soil pH levels greater than 7.0. In some soils, volatilization loss is highly temperature dependent (Liu et al. 2007). Volatilization risk is also high on lighter-textured soils with a low buffering capacity (Griggs et al. 2007).

#### 13.3.3.4 Nitrogen Immobilization

This N loss mechanism is more temporary in nature. Soil microbes that decompose high carbon-content plant residues to organic matter use soil N during the decomposition process (Killpack 1993). Consequently, the N from the surface-applied fertilizer is “tied up” in the resulting organic matter and is temporarily unavailable for plant uptake until mineralization of the organic matter occurs at a later date. Such immobilization of soil N can be especially prevalent in high-residue no-till cropping systems.

#### 13.3.3.5 N Loss through Aerial Part of Crop Plants

Cereal plants release N from plant tissue, predominantly as  $\text{NH}_3$  following anthesis (Francis et al. 1993). Plant N losses have accounted for 52%–73% of the unaccounted 15% N in corn (Francis et al. 1993), and between 21% (Harper et al. 1987) and 41% (Daigger et al. 1976) in winter wheat. Gaseous plant N loss in excess of  $45 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  has also been documented in soybean (Stutte et al. 1979).

The 90 day average plant emission of  $1.8 \text{ mg N m}^{-2} \text{ day}^{-1}$  represented a crop loss of  $1.6 \text{ kg N ha}^{-1}$  and 3.1% of the total N in the wheat plant contained in the chamber (O'Deen and Porter 1986).

The influence of different physiological and environmental parameters on  $\text{NH}_3$  exchange between plants and the atmosphere was reviewed by Schjoerring (1998, 2000). A central parameter in controlling the rate and direction of  $\text{NH}_3$  fluxes is the  $\text{NH}_3$  compensation point. It may vary from below 1 to over  $20 \text{ nmol NH}_3 \text{ mol}^{-1} \text{ air}$ . High compensation points seem to be a result of high tissue N status, rapid absorption of  $\text{NH}_4^+$  from the root medium, and/or low activity of glutamine synthetase, a key enzyme in  $\text{NH}_4^+$  assimilation. These conditions cause the  $\text{NH}_4^+$  concentration in leaf apoplast and leaf cells to increase. The  $\text{NH}_3$  compensation point also depends on the plant developmental stage with peaks in  $\text{NH}_3$  emission related to leaf senescence and N remobilization. The leaf temperature has a profound influence on the  $\text{NH}_3$  compensation point: an increase in temperature from  $15^\circ\text{C}$  to  $30^\circ\text{C}$  may cause a plant to switch from being a strong sink for atmospheric  $\text{NH}_3$  to being a

significant  $\text{NH}_3$  source. Stomatal conductance for  $\text{NH}_3$  relative to that of water vapor increases with the tissue N status and with leaf senescence. At a given leaf temperature, the  $\text{NH}_3$  compensation point can be successfully predicted on the basis of pH and  $\text{NH}_4^+$  concentration in the apoplast of the mesophyll cells. A study conducted with entire plants of spring wheat (*Triticum aestivum*) from tillering to maturation has revealed that the maximum emission of  $\text{NH}_3$  from a plant occurs during ear emergence to grain-filling period (Rroço and Mengel 2000).

#### 13.3.3.6 Factors Influencing N Loss

Nitrogen loss potential is influenced primarily by the type of crop (Cassman 2002), the type of N fertilizer applied (Nielsen 2006), the method of fertilizer application (Limon-Ortega et al. 2000, Lopez-Bellido 2006, Stevens et al. 2007, Barbieri et al. 2008, Rochette et al. 2009), the amount of applied fertilizer (Buresh et al. 2007), the type of soil (Wan et al. 2009), and the crop season (Vaio et al. 2008). Rainfall, sunshine, and temperature all influence the rate of volatilization of surface-applied urea-based products. The timing and amounts of rainfall influence the rates of leaching and denitrification losses of available nitrate-N.

The time of application of the fertilizer during crop growth plays a crucial role in determining the amount of N utilized. Dry matter and N accumulated by winter wheat (*Triticum* spp.) plants until anthesis are of importance under a Mediterranean climate, because the yield greatly depends on the translocation of pre-anthesis assimilates to the grain (Papakosta and Gagianas 1991). NUE of crops such as rice under irrigated condition can be affected by the form of N fertilizer used (Duan et al. 2007).

### 13.4 PROGRESS IN IMPROVING NUE OF CROP PLANTS

All the factors that influence the loss of fertilizer N and also the potential of crop to utilize the applied N across time and space determine the extent and possibility of enhancing N utilization by crop plants. Improved NUE can be achieved by fertilizer management that enhances fertilizer use efficiency by crop and also by improving the genetic potential of plants to take up and utilize the applied N. A lot of efforts have been devoted to improve the N fertilizer management that has shown great potential to reduce the N use without affecting the yield substantially under given conditions (Cassman 1998, Buresh 2007, Buresh et al. 2007). Recently, the improvement of the genetic potential of crops for N use is gaining importance. This can be achieved by a proper understanding of NUE components.

#### 13.4.1 COMPONENTS OF NUE

Numerous processes are involved in NUE in crop plants, and the concept for evaluating the contribution of N uptake and utilization processes to the variation in NUE have been developed and demonstrated in corn (Moll et al. 1982).

NUE is a complex trait. Moll et al. (1982) and Ortiz-Monasterio et al. (1997) showed that NUE comprises the N uptake efficiency (UPE) and the N utilization efficiency (UTE), the latter of which can be further split into biomass production efficiency (BPE) and harvest index (HI).

#### 13.4.2 GENETIC VARIABILITY FOR NUE IN CROP PLANTS

The recent plateau in the yield potential trend of majority of staple crops now has set high demands on plant breeders to develop cultivars not only with increased yield potential but also with higher NUE, thereby combining the improved ability to absorb N more efficiently from the soil with efficiency in utilizing it for biomass production and grain yield (GY).

Variability in NUE or its components between the crop plants and within the crop plants has been reported. A comparison of N harvest index (NHI) and N remobilization efficiency (NRE)



revealed that both were low for wheat, linking this with high N uptake after anthesis, suggesting that in wheat the proportion of the assimilated N used immediately in the developing grain is greater than in barley and oat. There was no strong N translocation from vegetative parts of the main shoots in wheat, which exhibited higher competition for N between vegetative and reproductive organs (Muurinen et al. 2007). This is in contrast with the earlier observations that in wheat, 60%–95% of the grain N comes from the remobilization of N stored in roots and shoots before anthesis (Palta and Fillery 1995, Habash et al. 2007). This might be due to the complexities associated with the type of soil and growth environments in which these experiments were conducted.

Genetic variability in NUE has been reported in maize (Moll et al. 1982, Coque and Gallais 2007), rice (Samonte et al. 2006, Duan et al. 2007), barley (Anbessa et al. 2009), canola (Gan et al. 2008), wheat (Brancourt-Hulmel, et al. 2003), and sorghum (Traore and Maranville 1999).

Results from experiments have shown genetic variation in cereals for UPE (Kelly et al. 1995, Singh and Arora 2001) and for UTE (Woodend et al. 1986, Papakosta 1994, Singh and Arora 2001). For wheat, UPE accounts for most of the variation in NUE at low N availability (Ortiz-Monasterio et al. 1997, Le Gouis et al. 2000). Recent results obtained under northern growing conditions showed that UPE has a stronger relationship with NUE than UTE (Muurinen et al. 2006). However, there were indications of differences between cereal species in their NUE to UTE relationship. The association was especially strong for oat (Muurinen et al. 2006), which occurs, as also noted by Isfan (1993), regardless of N supply. Furthermore, Delogu et al. (1998) showed that in low-N-input environments, winter barley had higher UTE than winter wheat and UTE was also associated with higher NHI. This indicates the retranslocation efficiency of N from vegetative plant parts to the grain. Many studies have indicated that 70% or more of the N harvested in seeds is derived from N remobilized from senescing vegetative plant parts (Austin et al. 1977, Cox et al. 1985, Papakosta and Gagianas 1991). Therefore, an improved understanding of plant N requirements and dynamics, particularly, BPE (biomass partition efficiency) and NRE (nitrogen remobilization efficiency) from vegetative parts among species and cultivars, is needed to determine better NUE.

### 13.4.3 GENETIC IMPROVEMENT

#### 13.4.3.1 Conventional Plant Breeding

Because the agronomic NUE is defined as the ratio of GY to N supplied (by soil and fertilizer), the GY potential under a given fertilizer level determines the NUE. Hence, breeders empirically select those that are more efficient in terms of N absorption and utilization. As modern maize genotypes were selected in the presence of high fertilization, they were consequently selected for their adaptation to a high input (Castleberry et al. 1984), though this assumption has been contradicted by results obtained in experiments with old and modern cultivars of wheat (Ortiz-Monasterio et al. 1997). However, the expression of genetic variability for GY is largely dependent on the level of N fertilization as evident from G (genotype) X N interaction in various experiments (Moll et al. 1987, Bertin and Gallais 2000).

#### 13.4.3.2 Quantitative Trait Loci for NUE

The improvement of crop yield has been possible through the indirect manipulation of QTLs that control the heritable variability of the traits and physiological mechanisms that determine biomass production and its partitioning. A critical analysis of how QTL-based approaches contribute to a better understanding of the genetic basis of crop performance has been explained elsewhere (Collins et al. 2008).

In temperate maize, Gallais and coworkers (Bertin and Gallais 2000, 2001, Gallais and Hirel 2004, Hirel et al. 2007) identified a set of QTLs for NUE and for GY and its components at high and low N levels. QTLs mapped in clusters and those identified under low N were generally a subset of those identified under high N, except for grain protein content, for which a higher number of QTLs were detected in low N. A number of genes that encode enzymes involved in N and C metabolism

were close to QTLs for vegetative development and for GY and its components (Gallais and Hirel 2004). These included genes for glutamine synthetase (GS; glutamine-ammonia ligase), sucrose-P synthase, sucrose synthase, and invertase ( $\beta$ -fructofuranosidase). The most notable outcomes of these studies were the colocation of a major GY QTL on chromosome 5 with the gene encoding cytosolic GS (*gln4* locus) and the correlation between the expression levels of the *gln4* alleles and the contributions of the respective QTL alleles at this locus. Other candidate genes encoding enzymes involved in N metabolism that collocated with NUE QTLs were the two GS genes (*gln1* and *gln2*) on chromosome 1 and the GS gene (*gln3*) on chromosome 4. Collectively, these results suggest that the increased productivity in maize genotypes under low N may be due to their ability to accumulate nitrate in the leaves during vegetative growth and to efficiently remobilize the stored nitrate during grain filling.

In tropical maize, QTLs for GY and secondary traits under varying N and water supply were identified by Ribaut et al. (2007). A mapping population previously developed for identifying QTLs under water-limited conditions was evaluated under varying N and water regimes. Two of the eight GY QTLs that were identified under low-N conditions were also detected under high-N conditions. Five QTLs were stable across the two low-N environments and five colocalized with QTLs identified for ASI (anthesis to silking interval) or for the number of ears per plant under low-N conditions. These results suggest that both ASI and the number of ears per plant can be selected simultaneously to improve the performance of maize under low N and drought stress (Ribaut et al. 2007). In wheat, a QTL meta-analysis and factorial regression were deployed to investigate QTL  $\times$  N interactions, revealing the influence of three major phenological trait loci, *Ppd-D1*, *Rht-B1*, and *B1*, on N-related QTLs (Laperche et al. 2007). Additionally, QTL clusters for GS activity on wheat chromosomes 2A and 4A coincided with the location of *GS* and *GSr* genes, respectively, and although QTL alleles for higher GS activity were associated with higher grain N, they showed little or no effects on GY components (Habash et al. 2007). QTLs for tolerance to low N have also been described in *Arabidopsis* (Loudet et al. 2003), rice (Lian et al. 2005), and barley (Mickelson et al. 2003).

### 13.4.3.3 Molecular Approaches for Improving NUE

From its inorganic origin to its biochemical destiny, N travels a long biological path that runs from soil to grains in the crop plants through numerous processes that operate in a coordinated way from uptake at root level to integration into the plant parts and grains after active N is released from applied N fertilizer in the rhizosphere. These processes are regulated at plant, cell, and molecular levels, and have been persistently explored to search avenues for improvement in NUE (Hirel et al. 2007, Pathak et al. 2008). The initial phase of molecular research for improved NUE in crop plants was based on the assumption that genes contributing to N uptake and N assimilation in plants are crucial. This has led to the accumulation of substantial knowledge on N uptake and assimilation mechanisms at the molecular level.

#### 13.4.3.3.1 Genes Associated with Regulation of N Uptake and Assimilation at Molecular Level

Plants take up and assimilate both nitrates and ammonium, with nitrate being the predominant form in most agricultural soils (Crawford and Glass 1998). Nitrate taken up by roots is then transported into cells via transporters from the NRT1 and NRT2 family of nitrate transporters (Forde 2000, Tsay et al. 2007). Once inside the cell, nitrate is reduced to nitrite by nitrate reductase (*NIA*), and then to ammonium by nitrite reductase (*NiR*). Ammonium is then assimilated into amino acids.

In addition to serving as a nutrient, nitrate also acts as a signal. When plants are first exposed to nitrate, genes in the nitrate assimilation pathway (*NRT*, *NIA*, *NiR*) are rapidly induced (Wang et al. 2007). Other genes, which are required for reprogramming carbon metabolism and providing chemical energy for reduction and assimilation, are also induced (Fritz et al. 2006). Transcriptome

analyses have shown that over 1500 genes are induced or repressed by nitrate within 20–180 min of treatment (Wang et al. 2007). Longer-term responses to nitrate include changes in root growth, development, and architecture, and in root-to-shoot ratios (Forde 2002, Walch-Liu et al. 2005, Walch-Liu et al. 2006). Enhanced Nicotinamide Adenine Dinucleotide Phosphate (NADP) (H) production due to the overexpression of *NADK2* (a chloroplastic Nicotinamide Adenine Dinucleotide (NAD) kinase) increased nitrogen assimilation. Glutamine and glutamate concentrations, as well as some other amino acids, were higher in the overexpressers. These results indicate that the overexpression of *NADK2* either directly or indirectly stimulates carbon (C) and N assimilation in *Arabidopsis* under restricted conditions (Takahashi et al. 2009).

*NRT1.1* mediates nitrate regulation of high-affinity nitrate uptake (Krouk et al. 2006). *NRT1.1* also controls the root colonization of nitrate-rich patches by a signaling pathway that may include *ANRI*, as both genes are expressed especially in root tips and *ANRI* derepression requires the *NRT1.1* function (Remans et al. 2006). A signaling role for *NRT1.1* is also supported by the finding that the nitrate reversal of glutamate inhibition of the primary root growth requires the *NRT1.1* function (Walch-Liu and Forde 2008, Forde and Walch-Liu 2009). The *NRT1.1* gene functions as a sensor in plants, which has been recently confirmed (Wang et al. 2009).

#### 13.4.3.3.2 Genes Associated with N Utilization

The regulatory mechanisms and genes responsible for N responses in plants have been investigated using genetics (Leydecker et al. 2000, Zhang and Forde 2000) and systems analysis (Gutierrez et al. 2005, Gutierrez et al. 2007). The ANR1 MADS (a DNA-binding protein conserved in plants, fungi, and animals) (Thomas 2001) box transcription factor, which controls lateral root branching in response to nitrate and is induced by N deprivation, was the first to be identified (Zhang and Forde 1998, Gan et al. 2005). A *Dof* (DNA binding with one finger) transcription factor was discovered that improves NUE at low N (Yanagisawa et al. 2004). More recent discoveries were the master clock control gene *CCA1*, which links organic N regulation and circadian rhythms (Gutierrez et al. 2008), and microRNA167, which mediates the cell-specific control of root development in response to N (Gifford et al. 2008). Most recently, a protein kinase, AtCIPK8, was identified that is needed for nitrate responses at high, but not at low, nitrate concentrations (Hu et al. 2009), and a DNA-binding protein, *AtNLP7*, which encodes the NIN-like protein 7 (NLP7), was found to function in the nitrate regulation of nitrate assimilation (Castaings et al. 2009). The *Arabidopsis* *NLP7* gene was recently shown to encode a nuclear-targeted protein that is needed for full nitrate induction of several nitrate-responsive genes (Castaings et al. 2009). *NLP7* mutants have altered root growth (longer primary roots and more lateral roots) typical of N-starved plants and are more resistant to water stress. However, this accumulated knowledge on the molecular aspects of N uptake is yet to be exploited for enhancing NUE in crop plants.

The most success in terms of increasing NUE through genetic modification has been through the overexpression in the roots of alanine aminotransferase (*AlaAT*), a downstream process in N assimilation (Good et al. 2007). In transgenic canola, yields were higher at low N associated with higher influx of N. In this material root alanine was increased and shoot glutamine decreased. Transgenic *Brassica napus* plants overexpressing a barley *AlaAT* cDNA, driven by a *Brassica* root-specific promoter (*btg26*), showed improved NUE. Compared with wild-type *Brassica*, transgenic plants showed increased biomass and seed yield in both the laboratory and field under low-N conditions, whereas no differences were observed under high-N conditions. These changes resulted in a 40% decrease in the amount of applied N fertilizer required under field conditions to achieve yields equivalent to those of wild-type plants (Good et al. 2007). A similar strategy was used for improving NUE in rice (Shrawat et al. 2008). Nipponbare, a model rice plant, was genetically engineered by introducing a barley *AlaAT* cDNA driven by a rice tissue-specific promoter (*OsAnt1*). This modification increased the biomass and GY significantly in comparison with control plants when plants were well supplied with N. Compared with controls, transgenic rice plants also demonstrated significant changes in key metabolites and total N content, indicating increased N UPE.

# 13.5 STRATEGIES AND OPPORTUNITIES FOR GENETIC IMPROVEMENT OF NUE

## 13.5.1 TARGETS FOR NUE IMPROVEMENT

### 13.5.1.1 Target Environments for NUE Improvement

The N response of a crop plant is highly influenced by the type of agro-eco environment, as plant growth and N loss mechanisms are largely determined by soil and climatic factors, as described in Section 13.4. This is also evident from an estimate of NUE across the regions, as presented in Figure 13.1. Hence, there should be a clear idea about the target environment for a particular crop. For the sake of simplicity, the target agro-eco environments for crop plants may be grouped into high-input environments and low-input environments. The high-input environments need a crop with high NUE to reduce the amount of fertilizer applied and enhance profit for farmers through a substantial reduction in the adverse impact of excess N on the environment. On the other hand, low-input environments need crops with NUE that can enable plants to take up and utilize limited N available for growth, development, and yield.

### 13.5.1.2 Realistic Target for Improved NUE and Its Possible Impact

There should be a realistic target for NUE for a particular crop for a particular agro-eco environment. For example, Brazil consumes about 150,000 tons of N fertilizers for rice cultivation, and the total N used to cultivate rice alone across the world is more than 17.4Mt, which amounts to 15%–17% of the total N use in agriculture (Heffer 2009). An optimistic research target of about 30% reductions in the applied N fertilizer without compromising the crop productivity can substantially benefit rice cultivation in the tropics. This is possible if NUE is enhanced from about 40%–57% (Figure 13.2).

### 13.5.1.3 Defined N-Threshold for Estimating Genetic Difference among Varieties

Crop plants, in general, differ in their ability to recover fertilizer N, and this is influenced by factors such as soil type and climatic conditions. Before initiating the experiments to determine genetic variability among the genotypes for NUE, it is necessary to determine the optimum level of fertilizer at which the plant yield is at its maximum for a given environment along with the recommended agronomic practices. The computation of fertilizer recovery should necessarily consider the native

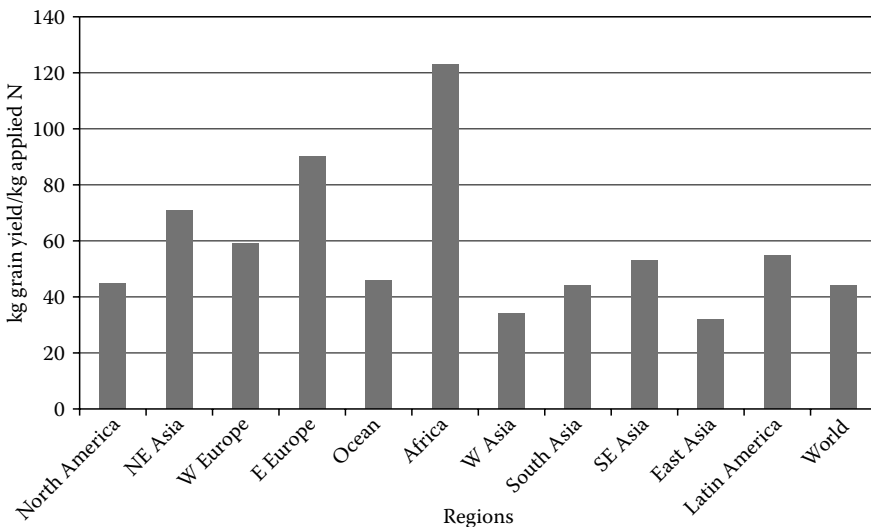
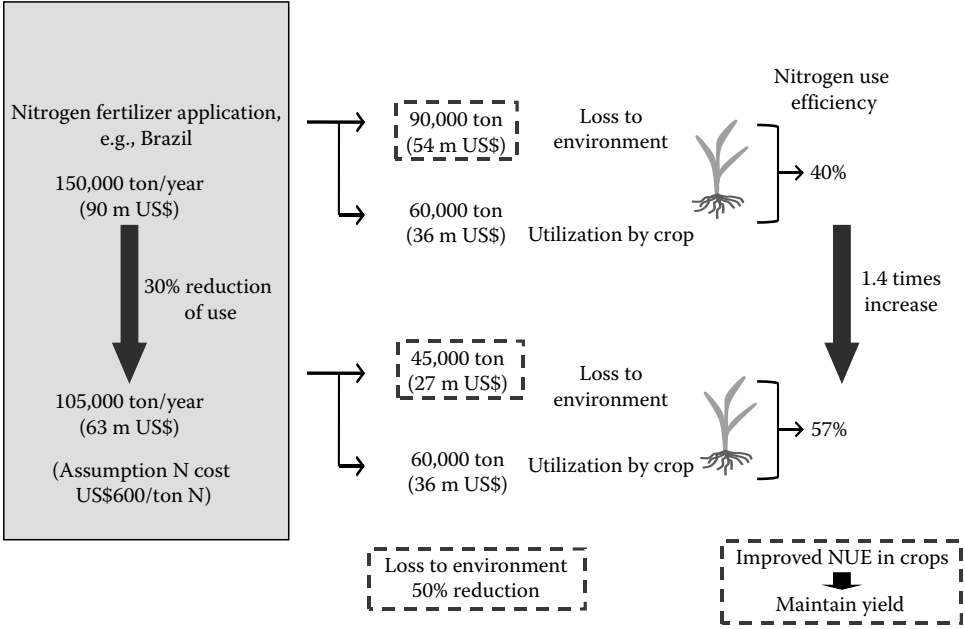
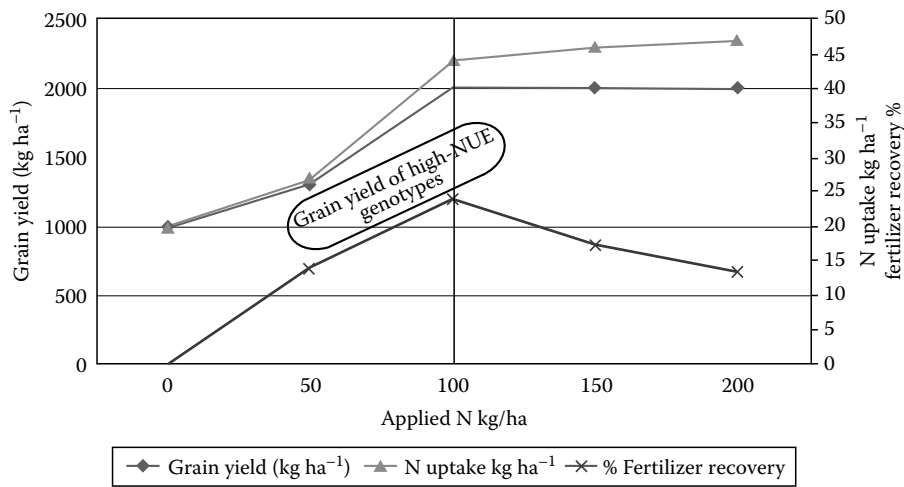


FIGURE 13.1 NUE of cereals across different regions. (From Doberman, 2006.)



**FIGURE 13.2** A hypothetical model showing the expected impacts of improvement in NUE in crop plants such as rice.



**FIGURE 13.3** A hypothetical N response curve and optimum N level for determining genetic variability in NUE.

N availability and its uptake by a check variety. For example, in Figure 13.3, it is clear that the benefits of additional fertilizer diminish beyond an N rate of 100 kg ha<sup>-1</sup>. So, the target for genetic improvement may be to develop a genotype that can produce more GY in response to the optimum N level, that is, 100 kg ha<sup>-1</sup> or the same level of GY at 50%–70% of the optimum fertilizer. This approach may be preceded by the use of the available crop model to estimate the optimum level of N to get the maximum yield under a particular environment. The validation of enhanced NUE through a molecular approach should consider the whole plant growth and development as well as GY in a given environment.

### 13.5.2 IMPROVING NUE COMPONENTS

#### 13.5.2.1 Improving N Uptake by Plant

In addition to the regulation of N supply by modern methods and conventional agronomic practices, there are also opportunities to genetically improve the N uptake by plant. This may be accomplished through an improvement in plant architecture in general and, specifically, the root (Garnett et al. 2009). Some of the suggested options to improve NUE through an improved root system were increased root-to-shoot ratio, high root vigor, N-induced root proliferation, root length density, root hairs, root exudates, microbial symbiosis, and root N metabolism. The utility of these traits in isolation or in combination is largely determined by the target crop environment.

As opined by Garnett et al. (2009), very little is known about how the various N transporters contribute to the net N uptake by crops in field situations or how this changes over the life of the crop. Furthermore, the mechanisms regulating the distribution of  $\text{NO}_3^-$  and/or  $\text{NH}_4^+$  from the root to the shoot remain poorly understood. Research in these areas will make it easier to focus on targets for improving NUE through increasing N UPE.

We now have evidence that all high-affinity  $\text{NO}_3^-$  transport systems appear to be induced by the presence of  $\text{NO}_3^-$ , and then down-regulated by the accumulation of endogenous N levels. This type of regulation exists even for an  $\text{NH}_4^+$  transporter. It is visualized that transcriptional and post-transcriptional regulation of events that follow the N uptake are essential to get benefit from the overexpression of high-affinity  $\text{NO}_3^-$  transporters, particularly at stages of plant development when both soil and plant N levels are abundant. This approach was successfully demonstrated by the overexpression of the *Dof1* transcription factor, which in *Arabidopsis* avoided the transport regulation processes and increased the N content of the plants by 30% (Yanagisawa et al. 2004).

#### 13.5.2.2 Improvement in N Utilization

Two possibilities related to the improvement of N distribution among plant organs have been suggested to increase crop productivity (Dreccer et al. 1998). The first one is to select cultivars with a greater capacity to store N in non-photosynthetic organs, such as internodes that allow the translocation of a larger amount of N to grains without reducing plant photosynthetic capacity (Martre et al. 2007). This is one trait associated with the “stay green” behavior, resulting in delayed leaf senescence and improved GY (Borrell et al. 2001). However, one needs to be careful as the gene for high grain protein content could accelerate senescence in leaves (Uauy et al. 2006). The second possibility is to improve the vertical distribution of N among leaves. Theoretical studies have suggested that canopy photosynthesis would be maximized if N is preferentially allocated to the more illuminated leaves (Field 1983). A vertical N distribution that follows the light gradient would allow higher photosynthesis compared with that expected from a uniform N distribution (Mooney and Gulmon 1979). The role of N dynamics on canopy photosynthesis and crop productivity will likely become even more important in the future because of climate change and the associated increase of atmospheric  $\text{CO}_2$  concentration (Kim et al. 2001, Anten et al. 2004).

### 13.5.3 MINIMIZING LOSS OF APPLIED N

Loss of applied N in the soil can be minimized through exploiting genetic variability in biological nitrification inhibition (BNI) by root exudates of a crop plant (Subbarao et al. 2006). Recently, an effective BNI has been discovered in the root exudates of the tropical forage grass *Brachiaria humidicola* (Rendle) Schweick (Subbarao et al. 2009). Named “brachialactone,” this inhibitor is a cyclic diterpene with a unique 5-8-5-membered ring system and a  $\gamma$ -lactone ring. It contributed 60%–90% of the inhibitory activity of exudates released from the roots of this tropical grass. Unlike nitrapyrin (a synthetic nitrification inhibitor), which affects only the ammonia monooxygenase (AMO) pathway, brachialactone appears to block both AMO and hydroxylamine oxidoreductase enzymatic pathways in *Nitrosomonas*. The release of this inhibitor is a regulated plant

function, triggered and sustained by the availability of ammonium ( $\text{NH}_4^+$ ) in the root environment. Brachialactone release is restricted to those roots that are directly exposed to  $\text{NH}_4^+$ . Within 3 years of establishment, *Brachiaria* pastures have suppressed soil nitrifier populations (determined as *amoA* genes; ammonia-oxidizing bacteria and ammonia-oxidizing archaea), along with nitrification and nitrous oxide emissions. These findings provided direct evidence for the existence and active regulation of a nitrification inhibitor (or inhibitors) release from tropical pasture root systems. Exploiting the BNI function could become a powerful strategy toward the development of low-nitrifying agronomic systems, benefiting both agriculture and the environment. The BNI activity of root exudates has been reported in *Brachiaria* and rice (Subbarao et al. 2007, 2009, CIAT 2008). However, long-term field experiments are needed to assess trade-offs between crop productivity and BNI, since the accumulation of compounds such as phenols in soil due to continuous rice cultivation may affect late season crop growth (Olk 2009).

#### 13.5.4 PHENOTYPING THE GENETIC RESOURCES AND MAPPING POPULATION

As described in Section 13.4.3.2, numerous attempts have been made to identify QTLs and genes associated with NUE; however, to the best of our knowledge, none of the discovered QTLs and genes have been applied successfully for a marker-aided selection for NUE in any of the crop in the public sector. This could be largely attributed to the complexity of NUE as a trait and the associated G X E interactions. It points out the need for gene or marker discovery based on precise high-throughput phenotyping for NUE under field and controlled environment.

A forward genetic approach that involves phenotyping followed by genotyping is the conventional way of associating a phenotype with the relevant gene. Attempts have been made earlier to screen germplasm for NUE; however, there is a need for scaling up this activity by utilizing recent advances in molecular techniques and phenomics. Reverse genetics that involves genotyping followed by phenotyping has recently become popular because of the access to DNA sequence data and information on gene function placed in public domain. This approach largely depends on the size of the target plant population screened as well as the accuracy of phenotyping. Hence, high-throughput screening is crucial to boost the reverse genetic approach for the discovery of genes that are associated with NUE. These techniques of phenomics can also accelerate phenotyping of diverse sets of genetic material that comprise germplasm collections; mutant libraries; mapping populations, such as recombinant inbred lines, advanced backcross lines, doubled haploid lines, and chromosome segment substitution lines (CSSLs); as well as minicore sets identified from germplasm collections. Phenomic techniques should not be restricted to control environments, but should also be adopted to screen a large number of plants under real field conditions. Appropriate criteria for designing phenotyping protocols in controlled and field environments are essential for improving NUE in cropping systems.

#### 13.6 FUTURE PERSPECTIVES

The major challenges to improve NUE include optimization of N supply and demand, maximization of crop N uptake, maximization of crop N assimilation, minimization of N losses, improvement in crop productivity, and interaction with other factors such as soil moisture. As shown in Table 13.2, there are great opportunities to address these challenges through agronomic, breeding, and molecular approaches. An efficient root system, BNI, and nitrate transporters may contribute to improved N UPE (Table 13.3). On the other hand, genetic manipulations leading to efficient C:N metabolism, enhanced efficiency of enzymes associated with N metabolism, and improved biomass partitioning to grain can contribute to improved NUE (Figure 13.4).

Genes and QTLs have been identified for NUE, but need to be validated in field conditions under different agro-eco environments. The significance of transgenes contributing to NUE has been demonstrated in the laboratories with model plants, such as *Arabidopsis* and *Nippombare*

**TABLE 13.2**  
**Strategies and Opportunities for Improving NUE in Crop Plants**

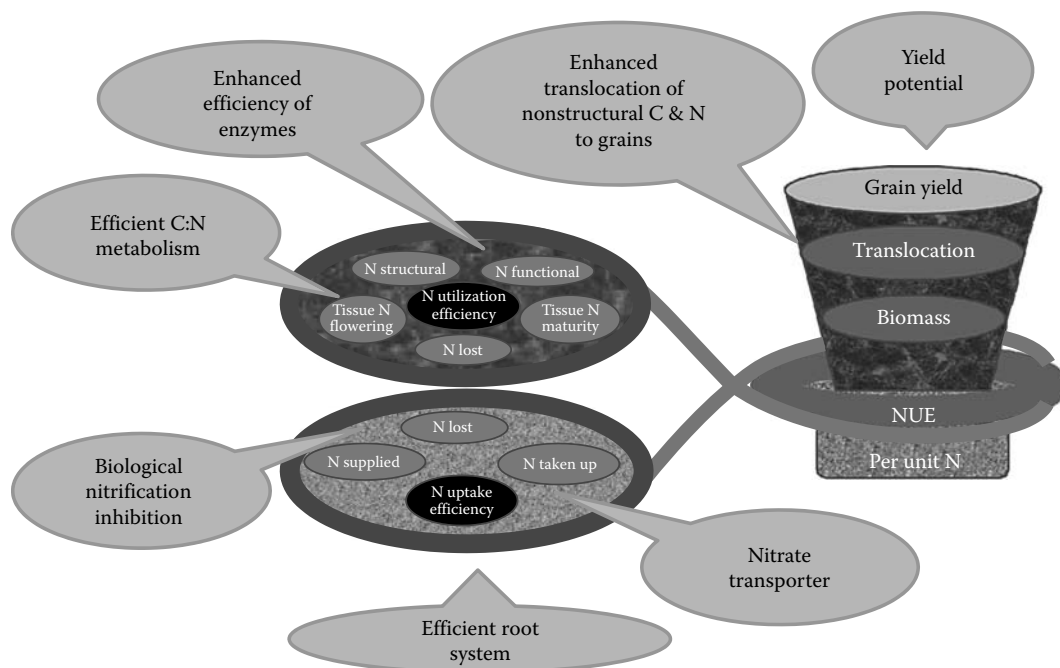
| Challenges                                   | Traits to be Improved                  | Agronomic Intervention                                                                                                                             | Genetic Improvement                   | Molecular Intervention                                                                                                                   |
|----------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Optimization of N supply                     | N UPE                                  | Split application; cover crop fertilization (Reiter et al. 2008); crop residue management (Reiter et al. 2008); precision agriculture (Table 13.3) | —                                     | —                                                                                                                                        |
| Maximization of crop N uptake                | N UPE                                  | Split application, precision agriculture                                                                                                           | Breeding for efficient root system    | Manipulation of nitrate transporters; control over internal regulation of N uptake; overcoming feedback inhibition (Garnett et al. 2009) |
| Minimization of N losses                     | N UPE, N UTE/ physiological efficiency | Slow-releasing fertilizers (Golden et al. 2009)                                                                                                    | Exploiting genetic variability in BNI | Genes associated with root architecture                                                                                                  |
| Maximization of crop N assimilation          | N UTE/ physiological efficiency        | —                                                                                                                                                  | —                                     | Manipulation of enzymes associated with N assimilation                                                                                   |
| Improvement in crop productivity             | Biomass partitioning                   | Recommended package of practices                                                                                                                   | Increased yield potential             | —                                                                                                                                        |
| Interaction with other factors such as water | Stacking traits of NUE + WUE+ yield    | Irrigation and soil management                                                                                                                     | Resistance to abiotic stress          | Manipulation of stress tolerance genes; gene stacking                                                                                    |

**TABLE 13.3**  
**Recent Agronomic Approaches for N Management**

| Technologies                                                                         | Description                                                                                                                                                                                                                                                  | References          |
|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| Sensor-based N application                                                           | Use of plant sensors–based diagnostic information for nitrogen recommendations                                                                                                                                                                               | Marek et al. (2009) |
| Automated calibration stamp technology for improved in-season nitrogen fertilization | Calibration stamps applied at preplant or soon after planting can assist in providing visual interpretation of N mineralization + atmospheric N deposition from planting to the time midseason N is applied, and improved determination of top dress N rates | Raun et al. (2005)  |
| In-season optical sensing                                                            | The optical sensor–based N management strategy is relatively easy to use, has better potential to improve NUE and economic returns, and reduces residual soil N content and apparent N loss                                                                  | Li et al. (2009)    |

(a rice variety) and also Brassica. In a review of the application of genomics to biotechnology traits, Gutterson and Zhang (2004) outlined a typical development process for an agricultural biotechnology product, which can take up to 12 years or more from gene discovery to a commercial product. The major challenge after discovering the lead in model plants, like Arabidopsis or a rice genotype such as Nipponbare, is its utility in the background of the commercial crop variety that is well adapted to the target environment. This technology needs to be evaluated under different environments with crop plants that are adapted to target environments with an appropriate phenotyping protocol.





**FIGURE 13.4** NUE components and possible approaches for improvement.

In addition to the conventional approach, advances in hyperspectral image-based phenomics are expected to accelerate plant phenotyping to a great extent (Furbank 2009). At present, many of these technologies are restricted to controlled environments, and are likely to be extended to field in the near future. This will enhance the capacity to screen genetic resources for new genes that are associated with NUE, and also will contribute to the validation of the technologies that are already applied in controlled environments.

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# 14 Photosynthesis and Light Stress in a Model Plant: Role of Chloroplast Transporters

*Cornelia Spetea and Benoît Schoefs*

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## 14.1 INTRODUCTION

Chloroplasts are crucial for plant cell metabolism since they perform a unique and complex process named photosynthesis. This process is also vital for aerobic and heterotrophic life on Earth, because it provides the carbohydrates that are sustaining every food chain. The light reactions of photosynthesis use very abundant resources on Earth, water, and sunlight, to produce oxygen and provide chemical energy (ATP) and reducing power (NADPH). These two compounds drive the dark reactions of photosynthesis, namely, CO<sub>2</sub> fixation into carbohydrates, but also the synthesis of many other essential components, including fatty acids, amino acids, and nucleic acids, all taking place



in the chloroplast stroma. The photosynthetic apparatus consists of four major complexes involved in the light reactions, namely, the water-oxidizing photosystem II (PSII), cytochrome *b<sub>6</sub>f* (cyt *b<sub>6</sub>f*), photosystem I (PSI), and the H<sup>+</sup>-translocating ATP synthase (CF<sub>0</sub>F<sub>1</sub>), all four located in the thylakoid membrane of cyanobacteria, algae, and higher plants. The first three photosynthetic complexes are involved in sunlight absorption, electron extraction, and transfer from water to NADP<sup>+</sup>, whereas the fourth complex uses the H<sup>+</sup> gradient created across the thylakoid membrane during electron transfer to power ATP synthesis.

Plants utilize sunlight to drive photosynthetic energy conversion, but this event may also cause inactivation of the photosynthetic apparatus (photoinhibition), primarily of the PSII complex, affecting the plant daily productivity (Kok, 1956; Jones and Kok, 1966a,b; Powles, 1984; Mattoo et al., 1989). The extent of photoinhibition is the result of a dynamic balance between photodamage and repair of PSII (Ohad et al., 1984; Aro et al., 1993; Aro et al., 2005). Therefore, photoinhibition occurs only under conditions when the rate of photodamage exceeds the rate of repair. To avoid photoinhibition, plants use photoprotective mechanisms (see Chapter 16) to either suppress the damage or to facilitate the repair of damaged PSII.

The repair of damaged photosynthetic machinery must be rigorously regulated, and crucial for this is an array of “auxiliary” proteins, such as kinases, phosphatases, proteases, stress-induced and heat-shock proteins, as well as solute and metabolite transporters. As compared to the available knowledge concerning photosynthetic machinery, there is still a lot to be done in the field of transport proteins to understand their importance for the photosynthetic process itself but also in the functioning of other plastid types. This chapter focuses on chloroplast transporters, which are specialized integral membrane proteins localized in the outer envelope, inner envelope, or in the thylakoid membrane. Their function is to exchange solutes and metabolites between the chloroplast stroma and cytosol or between the stroma and thylakoid lumenal space. The majority of the substrates are anions, e.g., adenine nucleotides, phosphate, sulfate, but some are cations, e.g., H<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, and Cu<sup>2+</sup>. All chloroplast transporters are nuclear-encoded proteins, and have to be imported and inserted into the envelope or the thylakoid membrane. Chloroplast transporters, described in this chapter, play an important role in biogenesis, optimal function, acclimation, and repair of the photosynthetic apparatus to ensure its efficiency under ever-changing and even stressful environmental conditions.

## 14.2 PHOTOSYNTHESIS, PHOTOACCLIMATION, AND PHOTOINHIBITION

Many structural and functional advances have been made during the past 10 years when it comes to the photosynthetic apparatus (Nelson and Ben-Shem, 2004; Merchant and Sawaya, 2005). A view of this amazing machinery at atomic resolution is a prerequisite for understanding the mechanism of its function and regulation. The crystal structures of the four major photosynthetic complexes have revealed their protein composition and interactions, and the arrangement of cofactors. In addition, they allow very refined models of the key features of their function, which will be the task for biophysicists and molecular biologists to validate.

PSII or water–plastoquinone oxidoreductase is the first complex in the photosynthetic electron transfer pathway. It is a membrane protein complex composed of over 25 different subunits and many functional cofactors, including chlorophylls, carotenoids, quinones, lipids, Cl<sup>−</sup>, and metal ions (Fe<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>), as revealed by several crystal structures of cyanobacterial PSII, published at increasing resolution (Kamiya and Shen, 2003; Ferreira et al., 2004; Guskov et al., 2009). Most of the active redox cofactors involved in the PSII electron transport are bound on the innermost subunit, namely, the reaction center D1 protein. The available crystal structures revealed that, similar to many other membrane protein complexes, the functional unit of PSII *in vivo* is a dimer, although recent reports propose instead the monomeric form (Takahashi et al., 2009; Watanabe et al., 2009). This finding would challenge the current models of PSII complex during light-induced damage–repair cycle, where the monomeric form has so far been considered only an intermediate form.



A multilevel network of adaptation/acclimation strategies exists to help plants to cope with fluctuations of intensity in the light environment, ensuring high levels of plant survival and productivity (Ruban, 2009). These levels can be divided as follows: organism level (slow, e.g., leaf orientation), cellular level (fast, e.g., chloroplast orientation), and molecular level (most profound, long-term: change in chlorophyll and chlorophyll-binding protein content; short-term: changes in the light-harvesting efficiency by state transitions, and thermal energy dissipation). A central role is played by the light-harvesting antenna complex (LHCII), which collects and (then) delivers the sunlight to the reaction center of PSII. The amount of delivered light is crucial because too much light triggers photoinhibition. Therefore, modulation of light harvesting has to be fine-tuned. Thermal energy dissipation is the major component of non-photochemical quenching (NPQ), and is triggered by the acidification of the thylakoid lumenal space (Oxborough and Horton, 1988). NPQ is localized within the LHCII antenna and not the PSII reaction center, and is dependent upon the presence of exclusively LHCII-bound xanthophylls, lutein, and zeaxanthin (Ruban and Horton, 1994; Niyogi et al., 2001). NPQ also requires the presence of the PsbS protein and the activity of the violaxanthin de-epoxidase cycle (Yamamoto et al., 1962; Li et al., 2002; see Chapter 16).

A sustained exposure to excess radiance will, however, lead inevitably to photoinhibition, and a decline in photosynthetic efficiency and productivity. The reaction center of PSII is the most susceptible to the photodamage due to the very strong oxidation potential of its reaction center chlorophyll (P680, 1.17 V) needed to oxidize water. If there are problems on the donor side, P680<sup>+</sup> lifetime will increase; this will oxidize pigments and amino acids, causing their degradation, and the subsequent degradation of the D1 protein. If the acceptor side is less efficient, this will lead to the formation of P680 triplet that will interact with oxygen and form singlet oxygen, which, in turn, will oxidize amino acids of the D1 protein, and lead to its subsequent degradation (Vass et al., 1992). Later on, using EPR technique, it has been demonstrated that distinct reactive oxygen species (ROS) are formed during the two types of photoinhibition, namely, hydroxyl radicals during the donor-side type and singlet oxygen during the acceptor-side type (Hideg et al., 1994). Plants survive photoinhibition through several mechanisms; among them, the PSII repair cycle includes D1 degradation, its *de novo* synthesis, and its reintegration into the reaction center. In a recent scheme, different from the “classical” one, Murata et al. (2007) proposes that the photodamage occurs primarily at the oxygen-evolving complex of PSII, and that high levels of intracellular ROS, such as H<sub>2</sub>O<sub>2</sub> and singlet oxygen, inhibit repair of PSII by suppressing *de novo* synthesis of the D1 protein.

Various types of environmental stresses, such as cold, heat, salt, and oxidative stress enhance the extent of photoinhibition. Originally, it has been assumed that this is a result of an enhanced damage to PSII, but most recent results show that the above-mentioned environmental factors increase the imbalance between photodamage and repair, since they produce ROS, which suppress D1 protein synthesis at the translational step (Takahashi and Murata, 2008). Another possibility proposed by the same authors is that the acceleration of photodamage is the result of an inhibition of photosynthetic CO<sub>2</sub> fixation by the above stress factors, which can regulate the generation of ROS.

In spite of considerable effort, our knowledge about the mechanism of D1 protein and PSII turnover during high light stress is still relatively fragmentary. Moreover, we know very little about the biogenesis and assembly of the photosynthetic apparatus under optimal growth conditions. It is known that the thylakoid membrane in higher plants has a characteristic structure since it is organized in appressed (grana) and non-appressed (stroma) thylakoids with distinct composition: grana thylakoids contain mainly PSII, while stroma lamellae are highly enriched in PSI and ATP synthase (Anderson and Andersson, 1982). Therefore, the complexes must acquire their correct location for assembly into functional units. The mechanisms involved in lateral trafficking of newly synthesized proteins into and from grana lamellae are not known. All polypeptides appear to integrate first into the stroma lamellae and perhaps also into the exposed grana regions, and migrate subsequently either as individual polypeptides or in assembled complexes to their final destination.

### 14.3 ARABIDOPSIS AS A MODEL PLANT IN PHOTOSYNTHESIS RESEARCH

The first published sequenced genome of a plant was that of *Arabidopsis thaliana*, almost 10 years ago (Arabidopsis Genome Initiative, 2000), and is used since then as a model organism in plant genomics research. After that, the genome sequences of two plants of economic importance, namely, rice (*Oryza sativa*) and poplar (*Populus trichocarpa*), and two model plants, *Medicago* (*Medicago truncatula*) and the moss (*Physcomitrella patens*) have also become available (International Rice Genome Sequencing Project, 2005; Tuskan et al., 2006; Retzel et al., 2007; Rensing et al., 2008). In addition, projects for sequencing many other plant genomes are in progress, according to the National Center for Biotechnology Information (NCBI) Entrez Genome projects website (<http://www.ncbi.nlm.nih.gov/>). These are either model plants or species of economic importance. References to organism-specific databases for the ongoing genome sequencing projects are provided in Table 14.1.

Among the sequenced plant genomes, *Arabidopsis* has the smallest plant genome (125 Mb) and a number of over 28,500 predicted genes (Arabidopsis Genome Initiative, 2000). For comparison, rice has around 41,000 genes, poplar more than 45,000 genes, and *Medicago* more than 40,000 genes (Sterck et al., 2007). Despite the rather small range of gene numbers, a large increase in the plant genome size has occurred during evolution and attributed to many duplication events. When the sequencing of *Arabidopsis* started, it had not been expected that this model plant with such a small genome could be an ancient polyploid. It has turned out that 60% of the genome is duplicated, and that

**TABLE 14.1**  
**Photosynthetic Organisms Used in Photosynthetic Research**

| Photosynthetic Organism                                   | Number of Publications<br>on Photosynthesis |       | Genome Sequencing Project |                                                     |
|-----------------------------------------------------------|---------------------------------------------|-------|---------------------------|-----------------------------------------------------|
|                                                           | 1950–                                       | 2000– | Stage                     | References                                          |
| <i>Synechocystis</i> ( <i>Synechocystis</i> sp. PCC 6803) | 427                                         | 283   | Complete 1996             | Kaneko et al. (1996)                                |
| <i>Arabidopsis</i> ( <i>Arabidopsis thaliana</i> )        | 958                                         | 765   | Complete 2000             | Arabidopsis Genome Initiative (2000)                |
| Rice ( <i>Oryza sativa</i> )                              | 366                                         | 311   | Complete 2005             | International Rice Genome Sequencing Project (2005) |
| <i>Medicago</i> ( <i>Medicago truncatula</i> )            | 39                                          | 27    | Complete 2006             | Retzel et al. (2007)                                |
| Poplar ( <i>Populus trichocarpa</i> )                     | 168                                         | 139   | Complete 2006             | Tuskan et al. (2006)                                |
| <i>Chlamydomonas</i> ( <i>Chlamydomonas reinhardtii</i> ) | 651                                         | 300   | Complete 2008             | Merchant et al. (2008)                              |
| Moss ( <i>Physcomitrella patens</i> )                     | 30                                          | 27    | Complete 2008             | Rensing et al. (2008)                               |
| Tobacco ( <i>Nicotiana tabacum</i> )                      | 517                                         | 317   | In progress               | Tobacco Genome Initiative                           |
| Maize ( <i>Zea mays</i> )                                 | 516                                         | 261   | In progress               | MaizeGDB                                            |
| Wheat ( <i>Triticum aestivum</i> )                        | 444                                         | 232   | In progress               | International Wheat Genome Sequencing Consortium    |
| Barley ( <i>Hordeum vulgare</i> )                         | 187                                         | 90    | In progress               | International Barley Genome Sequencing Consortium   |
| Tomato ( <i>Solanum lycopersicum</i> )                    | 157                                         | 105   | In progress               | International Tomato Sequencing project             |
| Pea ( <i>Pisum sativum</i> )                              | 296                                         | 126   | Not found                 | —                                                   |
| Spinach ( <i>Spinacea oleracea</i> )                      | 925                                         | 256   | Not found                 | —                                                   |

Note: The number of publications were taken from PubMed (<http://www.ncbi.nlm.nih.gov/sites/entrez>) (2009-11-11).

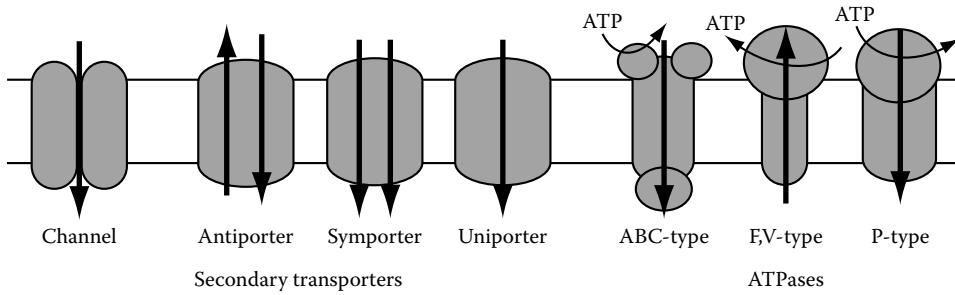
this is the result of three steps of duplication during 150–200 million years (Simillion et al., 2002). Another reason for the large genome size of land plants could be their unique lifestyle, since they are sessile, and thus need genes and strategies to acclimate to abiotic stress and to defend biotic stress.

Unraveling the molecular details of the photosynthetic reactions has been a central topic of research in biochemistry and biophysics. Among higher plants, spinach has been the model for biophysics, and especially for biochemistry of photosynthesis. However, the spinach genome has not been sequenced, and there are no plans to do so (Table 14.1). As in present life sciences in general, identifying sequences and mutating genes encoding specific proteins have revealed a vast amount of new knowledge, which could not be obtained using conventional biochemistry. Determination of the complete genome sequence of model photosynthetic organisms, such as the cyanobacterium *Synechocystis* sp. PCC 6803, the green algae *Chlamydomonas reinhardtii*, and the higher plant *A. thaliana*, has been a significant development.

The genome of *Synechocystis* sp. PCC 6803 has been sequenced by Kaneko et al. (2006). It is a prokaryotic oxygenic photosynthetic organism, used as a model for photosynthesis research because the reaction centers (RCs) are highly similar to those in plants. Its major limitation is that the light-harvesting system and the oxygen-evolving complex in *Synechocystis* are rather different from those in plants, and that the *cyt b<sub>6</sub>f* complex is part of both respiratory and photosynthetic electron transport chains. *C. reinhardtii* genome has been sequenced by Merchant et al. (2007). It has been used as a model for photosynthesis research for more than 50 years, and some of the reasons are listed below: ability to grow heterotrophically, ability to synthesize chlorophyll in darkness, and easiness to perform chloroplast transformation (Dent et al., 2001). *Arabidopsis* has become a model organism in plant genomics and also in photosynthesis research due to several factors: easy to cultivate, short life cycle, self-progeny, publicly available genome sequence, predicted sequence information, gene expression data, and collections of DNA and seeds (The Arabidopsis Information Resource Web site and associated links; <http://www.arabidopsis.org/>). The number of publications on photosynthesis found at NCBI Web site since 1950 is similar in *Arabidopsis* and spinach (Table 14.1). However, much more has been published using *Arabidopsis* as plant material since 2000, the year of its genome sequencing. Therefore, in the last decade, molecular genetics has provided a new and powerful tool in this area of research.

#### 14.4 PLANT MEMBRANE TRANSPORTERS: CLASSIFICATION AND STRATEGIES FOR IDENTIFICATION

According to the Transport Classification (T.C.) system available at the TCDB database (<http://www.tcdb.org/>), the membrane transport proteins found in biological membranes have been divided into three categories: channels/porins, secondary transporters, and primary transporters (pumps). The channels (T.C. #1) are non-energy consuming, since they transport down the gradient and are the fastest ( $10^7$ – $10^8$  molecules/s); the secondary transporters (T.C. #2) use the gradient of cotransported molecules for transport ( $10^2$ – $10^4$  molecules/s); and the pumps (T.C. #3) consume energy (mainly ATP) for transport, and are the slowest ( $1$ – $10^3$  molecules/s). The most common subtypes of these three categories are illustrated in Figure 14.1. The two main types of secondary transporters are antiporters (counter-transport) and symporters (cotransport). Symporters use the downhill movement of one solute species from high to low concentration to move another molecule uphill from low concentration to high concentration, i.e., against its electrochemical gradient. There is a third type of secondary transporters designated uniport, when a single species is transported either by facilitated diffusion or in a membrane-potential-dependent process, if the solute is charged. There are four types of ATP-utilizing pumps in biological membranes, namely, ATP-binding cassette (ABC) transporters,  $H^+$ -translocating F-ATPases, vacuolar V-ATPases, and metal ion-transporting P-ATPases. Pumps transport either molecules in specific directions independent of the environmental situation, or ions to build a concentration gradient between the outside and the inside of the membrane (i.e., active transport). Plants also contain transporters that so far could not be assigned to a certain category (T.C. #9).

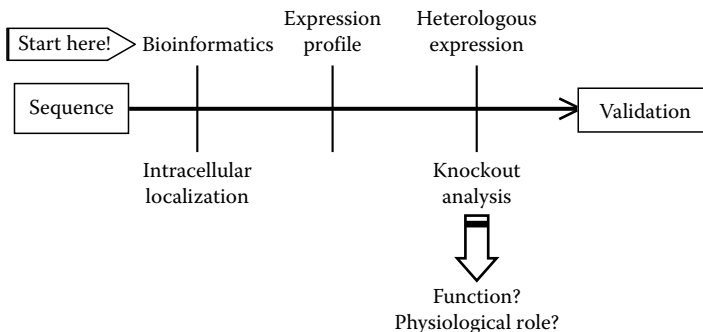


**FIGURE 14.1** Schematic illustration of the most common types of transporters in biological membranes.

Except for TCDB, there are several other excellent databases for membrane transporters, namely, TransportDB for genomic comparison of membrane transport systems (<http://www.membranetransport.org/>), PlantsT for functional genomics of plant transporters (<http://plantst.genomics.purdue.edu/>), and ARAMEMNON for sequence analysis of plant membrane proteins (<http://aramemnon.botanik.uni-koeln.de/>). Interestingly, the total number of membrane transporters in plants is 1.2 to 2.0-fold higher than in animals, and explained on the basis of their immobility (Nagata et al., 2008). The same report presents bioinformatics data revealing a smaller number of ion channels in plants than in animals, and at the same time a larger number of pumps and secondary transporters. The latter observation is explained by the fact that plants have chloroplast synthesizing ATP during photosynthesis, which is used by pumps to drive the transport of molecules, which in turn, act as the driving force of secondary transporters.

The major structural features of membrane transporters are a hydrophobic structure, a pore-forming sequence, and molecule-binding sites. Based on these specific features, homologues of known membrane transporters can be identified using bioinformatics. Following the sequencing of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), bioinformatics analyses have estimated that 5%–10% of the nuclear-encoded proteins represent membrane transporters (Weber et al., 2005). Most recently, Nagata et al. (2008) reported that *Arabidopsis* has around 1000 transporters, which represent 4.5% of its genome, and include 13% ion channels, 67% secondary transporters, and 18% ATP-dependent pumps.

As compared to the knowledge we have concerning the photosynthetic machinery, there is still a lot to be done in the field of chloroplast transport proteins. First, there is need to identify them, and then to thoroughly characterize their function and physiological role. Proteomics has been a very successful approach in identifying a tremendous number of chloroplast proteins (Schröder and Kieselbach, 2003; Rolland et al., 2003; van Wijk, 2004). The identification using large-scale mass-spectrometry-based proteomics is hampered by the fact that, as regulatory proteins, they are present in amounts predicted to be  $10^7$ – $10^{14}$  fold lower than those for the photosynthetic proteins. The



**FIGURE 14.2** Schematic representation of *in silico* and experimental approaches used in functional genomics.

alternative is to mine the available sequence information of model plants such as *Arabidopsis*, and use a functional genomics approach (Figure 14.2; Barbier-Brygoo et al., 2001). This includes prediction of intracellular location and function using sequence-based bioinformatics tools, localization studies using green fluorescent protein-constructs and fluorescence microscopy, expression studies using quantitative RT-PCR, functional studies using heterologous expression and activity assays, and phenotypic analyses of knockout mutants.

14.5 CHLOROPLAST TRANSPORTERS

Chloroplast membrane proteins belong to one of the following three compartments: outer envelope, inner envelope, or thylakoid membrane. These chloroplast membranes are distinct subcellular compartments in several aspects. The outer chloroplast envelope membrane has been thought for a long time to be freely permeable to solutes, while the inner envelope and the thylakoid membrane function as selective barriers. However, several transporters (e.g., porins) have been most recently found in the outer envelope membrane (Duy et al., 2007a). Chloroplast transporters belonging to channels, secondary transporters, pumps, and so far unclassified transporters are reviewed in this chapter. Tables 14.2 through 14.4 include accession numbers for *Arabidopsis* members, together with their experimentally validated location, substrate specificity, and physiological role. Figure 14.3 provides an overview of the transporters so far identified and characterized in the chloroplast thylakoid membrane of *Arabidopsis*.

TABLE 14.2  
Chloroplast Channels and Porins from *Arabidopsis thaliana*

| Family Name<br>(Chloroplast Members)          | AGI Number | Location                           | Substrate<br>Specificity                                       | Physiological Relevance                                      |
|-----------------------------------------------|------------|------------------------------------|----------------------------------------------------------------|--------------------------------------------------------------|
| <b>1.A. <math>\alpha</math>-Type channels</b> |            |                                    |                                                                |                                                              |
| Chloride channel<br>(AtCLCe)                  | At4g35440  | Thylakoid                          | Chloride, nitrite                                              | Photosynthesis, nitrate homeostasis                          |
| CO <sub>2</sub> aquaporin<br>(NtAQP1)         | —          | Plasma membrane/<br>inner envelope | CO <sub>2</sub> , water                                        | Leaf internal CO <sub>2</sub> conductance and photosynthesis |
| Inner envelope Tic110<br>(AtTic110)           | At1g06950  | Inner envelope                     | Anion-selective channel                                        | Import of nuclear-encoded preproteins in plastids            |
| Mg <sup>2+</sup> channel<br>(AtMRS2-11)       | At5g22830  | Inner envelope                     | Mg <sup>2+</sup>                                               | Not clear                                                    |
| <b>1.B. <math>\beta</math>-Type porins</b>    |            |                                    |                                                                |                                                              |
| Outer envelope protein 16 (PsOEP16)           | —          | Outer envelope                     | Cation-selective: amino acids, primary amines                  | Transport of solutes and metabolites into plastids           |
| Outer envelope protein 21 (PsOEP21)           | —          |                                    | Anion-selective                                                |                                                              |
| Outer envelope protein 24 (PsOEP24)           | —          |                                    | Triose-phosphate, ATP, PPI, dicarboxylate, charged amino acids |                                                              |
| Outer envelope protein 37 (AtOEP37)           | At2g43950  |                                    | Cation-selective                                               | Embryogenesis and germination                                |

Note: The classification is according to the Transport Classification Database (<http://www.tcdb.org/>). The accession numbers in *Arabidopsis* (Arabidopsis gene index, AGI) is indicated. n.d., not determined. The source of these data is given in the text describing each chloroplast member.

**TABLE 14.3**  
**Chloroplast Secondary Transporters from *Arabidopsis thaliana***

| Family Name<br>(Chloroplast Members)                                 | AGI Number | Location       | Substrate<br>Specificity                                    | Physiological Relevance                                                           |
|----------------------------------------------------------------------|------------|----------------|-------------------------------------------------------------|-----------------------------------------------------------------------------------|
| 2.A.1. Major facilitator (MFS)                                       |            |                |                                                             |                                                                                   |
| Anion transporter 1 (AtPHT4;1)                                       | At2g29650  | Thylakoid      | Pi                                                          | PSII repair and photoprotection<br>n.d.                                           |
| Anion transporter 2 (AtPHT4;4)                                       | At4g00370  | Inner envelope | Pi                                                          |                                                                                   |
| 2.A.7. Drug/metabolite transporter (DMT)                             |            |                |                                                             |                                                                                   |
| Triose-phosphate/phosphate translocator (AtTPT)                      | At5g46110  | Inner envelope | TPT, Pi                                                     | Sucrose and cell wall biosynthesis                                                |
| Phosphoenolpyruvate/Phosphate transporter (AtPPT1)                   | At5g33320  |                | PEP, Pi                                                     | Fatty acid synthesis and shikimate pathway                                        |
| AtPPT2                                                               | At3g01550  |                |                                                             |                                                                                   |
| 2.A.20. Inorganic phosphate translocator (PiT)                       |            |                |                                                             |                                                                                   |
| Inorganic phosphate 2 translocator (AtPHT2;1)                        | At3g26570  | Inner envelope | H <sup>+</sup> , Pi                                         | Pi homeostasis in green and non-green tissues                                     |
| 2.A.12. ATP/ADP antiporters (AAA)                                    |            |                |                                                             |                                                                                   |
| ATP/ADP antiporter 1 (AtNTT1)                                        | At1g80300  | Inner envelope | ATP, ADP                                                    | Reprogram chloroplasts into starch-storing plastids                               |
| ATP/ADP antiporter 2 (AtNTT2)                                        | At1g15500  |                |                                                             | Chlorophyll biosynthesis and protein import                                       |
| 2.A.29. Mitochondrial carriers (MC)                                  |            |                |                                                             |                                                                                   |
| Thylakoid ATP/ADP carrier (AtTAAC)                                   | At5g01500  | Thylakoid      | ATP, ADP                                                    | Thylakoid biogenesis, PSII repair, and photoprotection                            |
| Adenine nucleotide uniporter Brittle-1 (AtBT1)                       | At4g32400  | N.D.           | ADP-glucose and ADP (cereals)<br>AMP, ADP, and ATP (potato) | Starch synthesis (cereals), export of adenine nucleotides to the cytosol (potato) |
| NAD <sup>+</sup> carrier (AtNDT1)                                    | At2g47490  | Inner envelope | NAD <sup>+</sup> , ADP, AMP                                 | NAD <sup>+</sup> -dependent pathways and redox balance in chloroplasts            |
| 2.A.37. Monovalent cation:proton antiporter 2 (CPA2)                 |            |                |                                                             |                                                                                   |
| Na <sup>+</sup> (K <sup>+</sup> )H <sup>+</sup> antiporter (AtCXH23) | At1g05580  | Inner envelope | Na <sup>+</sup> , K <sup>+</sup> , H <sup>+</sup>           | pH and ion homeostasis                                                            |

*Note:* The classification is according to the Transport Classification Database (<http://www.tcdb.org/>). The accession numbers in *Arabidopsis* (Arabidopsis gene index, AGI) is indicated. n.d., not determined. The source of these data is given in the text describing each chloroplast member.

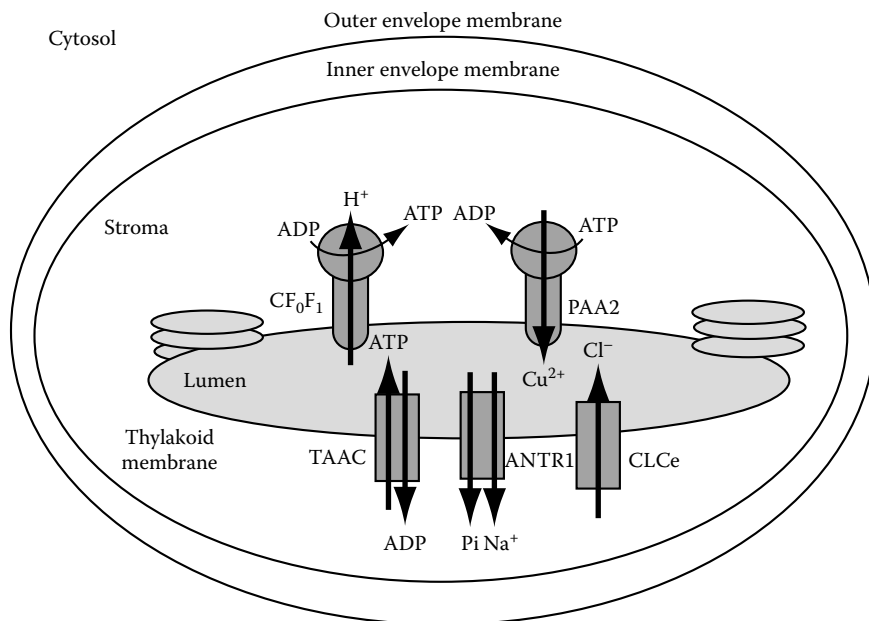
### 14.5.1 CHANNELS/PORINS

Channels/porins (T.C. #1) catalyze facilitated diffusion by passage through a transmembrane aqueous pore or channel without evidence for a carrier-mediated mechanism. Channels are the fastest at transporting molecules down their concentration gradient without consuming energy. There are five different types, which include  $\alpha$ -type channels (T.C. #1.A) and  $\beta$ -barrel porins (T.C. #1.B). The  $\alpha$ -channel proteins usually consist largely of  $\alpha$ -helical spanners, although  $\beta$ -strands may be

**TABLE 14.4**  
**Chloroplast Pumps and Unclassified Transporters from *Arabidopsis thaliana***

| Family Name<br>(Chloroplast Members)                              | AGI<br>Number | Location       | Substrate<br>Specificity                                                  | Physiological Relevance                                                                  |
|-------------------------------------------------------------------|---------------|----------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| 3.A.1. ATP-binding cassette (ABC) transporters                    |               |                |                                                                           |                                                                                          |
| Pleiotropic drug resistance ABC transporter (AtPDR7)              | At1g15210     | Inner envelope | n.d.                                                                      | n.d.                                                                                     |
| AtPDR8                                                            | At1g59870     |                |                                                                           |                                                                                          |
| ABC transporter homologue 12 (AtATH12)                            | At5g03910     |                |                                                                           |                                                                                          |
| 3.A.2. H <sup>+</sup> -translocating F-type ATPase (F-ATPase)     |               |                |                                                                           |                                                                                          |
| ATPase alpha subunit, CF <sub>1</sub> —atpA                       | AtCg00120     | Thylakoid      | ATP                                                                       | ATP supply for CO <sub>2</sub> fixation and other energy-dependent chloroplast processes |
| ATPase beta subunit, CF <sub>1</sub> —atpB                        | AtCg00480     |                | H <sup>+</sup>                                                            |                                                                                          |
| ATPase gamma subunit, CF <sub>1</sub> —atpC                       | At4g04640     |                |                                                                           |                                                                                          |
| ATPase delta subunit, CF <sub>1</sub> —atpD                       | At4g09650     |                |                                                                           |                                                                                          |
| ATPase epsilon subunit, CF <sub>1</sub> —atpE                     | AtCg00470     |                |                                                                           |                                                                                          |
| ATPase I subunit, CF <sub>0</sub> -I—atpF                         | AtCg00130     |                |                                                                           |                                                                                          |
| ATPase II subunit, CF <sub>0</sub> -II—atpG                       | At4g32260     |                |                                                                           |                                                                                          |
| ATPase III subunit CF <sub>0</sub> -III—atpH                      | AtCg00140     |                |                                                                           |                                                                                          |
| ATPase a subunit, CF <sub>0</sub> -IV—atpI                        | AtCg00150     |                |                                                                           |                                                                                          |
| 3.A.3. P-type ATPases (P-ATPase)                                  |               |                |                                                                           |                                                                                          |
| Cu <sup>2+</sup> -transporting ATPase (AtPAA1)                    | At4g33520     | Inner envelope | Cu <sup>2+</sup>                                                          | Copper supply into the stroma                                                            |
| Cu <sup>2+</sup> -transporting ATPase (AtPAA2)                    | At5g21930     | Thylakoid      | Cu <sup>2+</sup>                                                          | Copper supply to the thylakoid lumen                                                     |
| Cu <sup>2+</sup> -transporting ATPase (AtHMA1)                    | At4g37270     | Inner envelope | Cu <sup>2+</sup> , Ca <sup>2+</sup> , Zn <sup>2+</sup>                    | Detoxification of excess zinc (II)                                                       |
| Calmodulin-binding Ca <sup>2+</sup> -transporting ATPase (AtACA1) | At1g27770     | Inner envelope | Ca <sup>2+</sup>                                                          | Chloroplast metabolism and signaling to cytosol                                          |
| 3.F.2. Photosynthetic reaction center (PRC)                       |               |                |                                                                           |                                                                                          |
| Photosystem I D1 protein                                          | AtC00020      | Thylakoid      | Mn, Chl, Fe <sup>2+</sup> , Q <sub>B</sub>                                | PSII electron transport                                                                  |
| Photosystem II D2 protein                                         | AtC00270      | Thylakoid      | Chl, Fe <sup>2+</sup> , Q <sub>A</sub>                                    |                                                                                          |
| 9.A. Unclassified transporters                                    |               |                |                                                                           |                                                                                          |
| Permease in chloroplast 1 (AtPIC1/AtTic21)                        | At2g15290     | Inner envelope | Fe <sup>2+</sup> , Cu <sup>2+</sup> , Mn <sup>2+</sup> , Zn <sup>2+</sup> | Chloroplast iron transport and homeostasis, chloroplast protein import                   |
| Putative iron-regulated transporter (AtMAR1/AtIREG3)              | At5g26820     | Inner envelope | Aminoglycoside antibiotics                                                | Cellular iron homeostasis                                                                |

*Note:* The classification is according to the Transport Classification Database (<http://www.tcdb.org/>). The accession numbers in Arabidopsis (Arabidopsis gene index, AGI) is indicated. n.d., not determined. The source of these data is given in the text describing each chloroplast member.



**FIGURE 14.3** Overview of transporters identified from the thylakoid membrane of *Arabidopsis thaliana*. Chloroplasts are structurally organized in three membrane compartments (outer envelope, inner envelope, and thylakoid membrane), and two soluble compartments (stroma and thylakoid lumen). The diagram shows the following transporters that have been localized to the thylakoid membrane and functionally characterized: the  $H^+$ -translocating ATPase ( $CF_0F_1$ ), the  $Cu^{2+}$ -transporting P-type ATPase (PAA2), the thylakoid ATP/ADP carrier (TAAC), the  $Na^+$ -dependent  $P_i$  transporter (ANTR1), and the chloride channel CLCe.

present and may even contribute to the channel. They are ubiquitously found in the membranes of all types of organisms from bacteria to higher eukaryotes. The transmembrane portions of porins consist exclusively of  $\beta$ -strands, which form a  $\beta$ -barrel. These proteins are found in the outer membranes of Gram-negative bacteria, mitochondria, and plastids. Chloroplast channels and porins are described below and summarized in Table 14.2.

Ion channels belong to the  $\alpha$ -type and regulate the flow of ions across the membrane in all cells. They conduct a specific species of ion, such as  $Na^+$  or  $K^+$ , and convey them through water-filled pores nearly as quickly as the ions move through free fluid. In some ion channels, passage through the pore is governed by a “gate,” which may be opened or closed by chemical or electrical signals, temperature, or mechanical force, depending on the channel type. Ion channels may be classified by the nature of their gating (i.e., what opens and closes the channels), the species of ions passing through those gates, and the number of gates (pores). Voltage-gated ion channels (VIC) activate/inactivate depending on the voltage gradient across the plasma membrane, while ligand-gated ion channels activate/inactivate depending on binding of ligands to the channel.

#### 14.5.1.1 Voltage-Gated Ion Channels

VIC (T.C. #1.A) is probably the largest subfamily of channels. The functionally characterized members are specific for  $K^+$ ,  $Na^+$ , and  $Ca^{2+}$  ions. Proteins of the VIC family are ion-selective channel proteins found in a wide range of bacteria, archaea, eukaryotes, and viruses. There are five members of this family predicted as chloroplast proteins (Weber et al., 2005). However, none of them has been so far characterized or identified in chloroplasts.

During photosynthetic light reactions, an electrochemical  $H^+$  gradient is generated between the chloroplast stroma and the thylakoid lumen, which is primarily used by the  $CF_0F_1$  to drive ATP synthesis. Evidence has been provided for the operation of ion channels in series with the electron



transfer chain (Cruz et al., 2001), which would electrically balance light-driven  $H^+$  transport. It is, indeed, well established by now that mainly  $K^+$ ,  $Mg^{2+}$ , and small anions take part in counterbalancing, and that changes in the concentrations of all these ions in the stroma are accompanied by passive  $H^+$ ,  $K^+$ ,  $Cl^-$ , and probably  $Na^+$  transport across the envelope of intact chloroplasts.

The activity of various channels has been reported using electrophysiology in the outer envelope, inner envelope, and the thylakoid membrane (Segalla et al., 2005 and references therein). This includes  $Cl^-$ ,  $K^+$ , divalent cation-selective and light-induced channels. By allowing specific ion fluxes across plastidial membranes, these ion channels may either directly or indirectly regulate photosynthesis. Segalla et al. (2005) also described the effects of various channel modulators on photosynthetic oxygen evolution, when added to isolated chloroplasts or thylakoid membranes. The observed effects were only modest, and are explained by the fact that a complete inhibition of photosynthesis cannot be expected even by completely inhibiting channel activity, since these channels are not part of the photosynthetic machinery itself and their function probably is not mandatory for photosynthesis to occur. The activity of a gated  $K^+$  channel has been reported in the thylakoid membrane (Tester and Blatt, 1989; Fang et al., 1995). This protein was supposed to mediate  $K^+$  efflux from the thylakoid lumen to charge balance the light-induced  $H^+$  gradient across the membrane. Its activity has been shown to be required for optimal  $O_2$  evolution, and to be regulated via ATP-dependent phosphorylation rather than voltage.

In animal and plant cells, anion channels/transporters play a role in the maintenance of electrochemical gradient and in signaling pathways allowing adaptation to stress of both biotic and abiotic type. They play various physiological roles in stomata opening, plant–pathogen interaction, compartmentation of metabolites and coupling with  $H^+$  gradient (De Angeli et al., 2007, 2009a). Channels from the plasma membrane are best characterized, using electrophysiological techniques, but, in most cases, the proteins responsible are not identified. Chloride channel (CLC) family comprises seven members in *Arabidopsis*, present in various membrane compartments, namely, vacuoles (AtCLCa), Golgi (AtCLCd and AtCLCf), and thylakoid membrane (AtCLCe) (Hechenberger et al., 1996; De Angeli et al., 2009a). The vacuolar AtCLCa member was shown to function as a  $2NO_3^-/H^+$  exchanger, and therefore, proposed to play a role in nitrate homeostasis (Geelen et al., 2000). Most recently, it has been reported that ATP binding to this protein regulates its transport activity (De Angeli et al., 2009b). The AtCLCe member is targeted to the thylakoid membranes in chloroplasts (Marmagne et al., 2007). In agreement with this subcellular localization, AtCLCe expression is higher in green tissues compared with roots, and the *clce* mutants display a phenotype related to photosynthetic activity, namely, a slower polyphasic chlorophyll fluorescence induction. Although no direct evidence for anion currents in *Arabidopsis* photosynthetic thylakoid membranes has been provided so far, anion channel activities have been previously reported on thylakoid membranes by Schönknecht et al. (1988) in the higher plant *Peperomia metallica*, and by Potossin and Schönknecht (1995, 1996) in the alga *Nitellopsis obtusa*. Changes in the intra-thylakoid ionic status are expected to occur in *clce* mutant, which could indirectly alter the kinetics of fluorescence induction. A direct effect is expected on the overall architecture of the thylakoid, since the formation of the grana stacks is known to depend on the ionic strength.

AtCLCe has been predicted to function as a voltage-gated CLC in thylakoids (Figure 14.3), supporting very specific biological functions, but not anion accumulation. Most recently, Monachello et al. (2009) proposed that, in chloroplasts,  $Cl^-$  and, most probably  $NO_2^-$ , are good candidates as counter-anions able to compensate for the excess positive charges in the thylakoid lumen. In this situation, alterations in ionic strength or osmotic properties of chloroplast compartments may, in turn, directly or indirectly increase the nitrite level, most probably in the cytosol, as exhibited by both *clce* and *clca* mutants. This might suggest a role for AtCLCe in nitrite translocation from the stroma into the thylakoids, taking over from the nitrite transporter of the chloroplast envelope (Sugiura et al., 2007). Analyses of T-DNA insertion *clca* and *clce* mutants revealed common phenotypic traits, including a lower endogenous nitrate content, a higher nitrite content, a reduced nitrate influx into the root, and a decreased expression of several genes encoding nitrate transporters (Monachello

et al., 2009). This set of nitrate-related phenotypes point to interconnecting roles of AtCICa and AtCICe in nitrate homeostasis, involving two different endocellular membranes.

#### 14.5.1.2 Aquaporins

A total of 35 aquaporins (AQP, T.C. #1.A.8), also known as major intrinsic proteins (MIP) have been identified in *Arabidopsis* by genome and transcriptome analysis, as compared to vertebrates for example where only 11–13 aquaporins exist (Kaldenhoff and Fischer, 2006; Wudick et al., 2009). Aquaporins transport water, but in some cases are also permeable for glycerol, urea, amino acids, and ions. Most recently, an aquaporin-sharing homology with the human aquaporin AQP1 has been identified in *Nicotiana tabacum*, and named NtAQP1. This protein has been initially localized to the plasma membrane, demonstrated to have a CO<sub>2</sub> transport function in *Xenopus* oocytes in addition to the water transport function, and shown that its high expression stimulates leaf photosynthesis and plant growth (Uehlein et al., 2003). Because of its location at the plasma membrane, NtAQP1 has been classified as a member of the plasma membrane intrinsic protein (PIPI) family, and it has been assumed that its function in CO<sub>2</sub> transport takes place in this membrane. This view has been revisited by Uehlein et al. (2008). Using immunogold localization of the native protein, and fluorescence microscopy of transiently expressed NtAQP1–GFP, these authors deduced localization for the protein at the plasma and inner envelope chloroplast membranes of tobacco mesophyll cells. They proposed that the dual localization of NtAQP1 is accompanied by a functional switch from a water channel (at the plasma membrane) to a CO<sub>2</sub> channel (at the chloroplast envelope). Thus, the envelope NtAQP1 has an important physiological function by facilitating CO<sub>2</sub> transport through the leaf to the site of CO<sub>2</sub> fixation in the stroma. Using RNA interference approach, it has been shown that plants with reduced expression levels of NtAQP1 increase by 90% the resistance to CO<sub>2</sub> of chloroplast membranes, hence reduce the rate of photosynthesis. These findings have challenged the concept of free diffusion of CO<sub>2</sub> through biological membranes, and expanded the substrate specificity of aquaporins to gases in an organelle where CO<sub>2</sub> plays the role of a substrate.

Several AQPs, including tonoplast intrinsic proteins AtTIP1;1, and TIP2;1 and plasma membrane intrinsic protein AtPIPI;1, have also been detected in a proteomic analysis of the *Arabidopsis* chloroplast envelope (Ferro et al., 2003). Although purification procedures for *Arabidopsis* chloroplasts have been highly optimized, these proteins were initially considered as extra-plastidial contaminants (e.g., Beebo et al., 2009). Given the recent data on the dual localization of NtAQP1, it needs to be reexamined whether the proteomic detection of those TIP and PIP proteins reflects their actual presence in chloroplast membranes.

#### 14.5.1.3 Translocon of the Inner Envelope Anion Channel 110 (Tic110)

The Tic110 protein of the inner chloroplast envelope membrane (T.C. #1.A.18) has been proposed to be a channel-forming protein at the inner envelope of chloroplasts, whose function is essential for the import of proteins synthesized in the cytosol. Tic110 appears to be the protein-import-related anion-selective channel (van den Wijngaard and Vredenberg, 1999). Most recently, its topology has been revealed as well as its regulation by stromal Ca<sup>2+</sup> ions and stromal thioredoxin (Balsera et al., 2009). Phenotypic analysis of knock-down mutants indicated a disturbed assembly of the Tic complex and reduction in the levels of a wide variety of plastid proteins (Inaba et al., 2005). Knockout homozygous *tic110* embryos exhibited retarded growth and arrest at the globular developmental stage, whereas heterozygous *tic110* plants exhibited chlorosis, aberrant chloroplast biogenesis, and inefficient chloroplast-import of both photosynthetic and non-photosynthetic preproteins (Kovacheva et al., 2005). Taken together, these data indicate an essential role for Tic110 in plastid biogenesis and plant viability.

#### 14.5.1.4 Mg<sup>2+</sup> Channel (MRS)

Magnesium (Mg<sup>2+</sup>) is essential for many plant processes. It is particularly important for photosynthesis, where it is the central element of the chlorophyll molecules. Besides this, Mg<sup>2+</sup> is also a

constituent of many enzymes and serves as an activator of others, including DNA polymerases, protein kinases, and phosphatases. Despite these critical cellular functions,  $Mg^{2+}$  uptake, transport, and homeostasis in eukaryotes are poorly understood at both the physiological and the molecular level.

CorA metal ion transporters (T.C. #1.A.35) represent a large and diverse family with sequenced members in Gram-positive and Gram-negative bacteria, cyanobacteria, archaea, plants, animals, and yeast. Their biochemical function is to transport  $Mg^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ , and  $Zn^{2+}$ , but not  $Fe^{2+}$  ions. The bacterial protein CorA, present in a wide range of bacterial species, is the best-characterized  $Mg^{2+}$  channel to date (Moomaw and Maguire, 2008). The CorA homologue MRS2 represents the mitochondrial  $Mg^{2+}$  channel in all eukaryotes, and is essential for cell survival. The *Arabidopsis* homologue MRS2-11 was localized to chloroplasts, and when expressed in yeast, it was shown to transport  $Mg^{2+}$  (Drummond et al., 2006). Using RT-PCR, it was demonstrated that the expression of this gene follows circadian rhythm, with a twofold higher steady-state expression in the light. No phenotype related to  $Mg^{2+}$  content and fluorescence yield was detected in plants over-accumulating the AtMRS2-11 protein. Therefore, no *in planta* role in  $Mg^{2+}$  homeostasis has been so far established for this gene.

#### 14.5.1.5 Outer Envelope Porins

The outer envelope membrane has long been assumed to be freely permeable for solutes with molecular weight of up to 10kDa. Correspondingly, it has been believed that the osmotic barrier against the cytosol is formed exclusively by the inner envelope. In Gram-negative bacteria, however, several different types of high-conductance channels exist in the outer membrane, such as porins, porin-like channels, and ligand-gated pores (Nikaido, 2003). The ancestral relation between plastids and Gram-negative bacteria, therefore, suggests the presence of multiple channel proteins in the chloroplast outer membrane. Indeed, the membrane-intrinsic outer envelope proteins (OEPs) form solute channels with properties reminiscent of porins and channels in the bacterial outer membrane. OEP channels are  $\beta$ -barell protein (T.C. #1.B.), and are characterized by distinct specificities for metabolites and a quite peculiar expression pattern in specialized plant organs and plastids, thus disproving the assumption that the outer envelope is a nonspecific molecular sieve (Duy et al., 2007a).

The  $\alpha$ -helical OEP16 forms a cation-selective high-conductance channel with a remarkable permeability for amino acids and amines. OEP16 from pea is best characterized, and shown to be voltage-sensitive, and impermeable to triose-phosphate and uncharged sugars (Pohlmeyer et al., 1997). In contrast, OEP21 has been shown to form an anion channel, which is regulated by ATP and triose-phosphate from the intermembrane space (Hemmler et al., 2006). The channel properties of OEP24, however, closely resemble those described for general diffusion pores. OEP24 allows the passage of triose-phosphate, ATP, PPI, dicarboxylate, and positively or negatively charged amino acids (Pohlmeyer et al., 1998). In summary, these findings indicate that the intermembrane space of chloroplasts is not freely accessible to low-molecular-weight solutes. Goetze et al. (2006) reported that OEP37 forms a rectifying, cation-selective, high-conductance channel, which is sensitive to peptides and may function during seed development and germination of *Arabidopsis* plants. Transcripts of AtOEP37 are ubiquitously expressed throughout plant development, and accumulate in early germinating seedlings as well as in late embryogenesis. However, phenotypic analyses of the knockout mutant *oep37-1* showed that the proper function of this single-copy gene is not essential for development of the mature plant. Thus, OEP37 may constitute a novel peptide-sensitive ion channel in the outer envelope of plastids with function during embryogenesis and germination.

### 14.5.2 SECONDARY TRANSPORTERS

Secondary or electrochemical-driven transporters (T.C. #2) work using the concentration gradient of cotransported molecules. The major facilitator superfamily (MFS, T.C. #2.A.1) represents the largest secondary transporter family (over 10,000 sequenced members) in all organisms including plants. Its members transport a wide variety of substrates, including carbohydrates, phosphates, amino acids, and cations. Protons and  $Na^+$  ions are commonly used as cotransported molecules by 80% of

the MFS members. If bacteria and plants mostly use  $H^+$ , animals use  $Na^+$  ions as the cotransported molecule. Nevertheless, there is an increasing number of  $Na^+$ -dependent transport systems described in plants, including phosphate transporters, organic acid transporters, and NhaD antiporter (Ruiz Pavón et al., 2008; Nagata et al., 2008). Most MFS members consist of 400–600 amino acid residues and possess either 12, 14, or 24 putative transmembrane  $\alpha$ -helical spanners. MFS antiporters operate *via* a single binding site, and an alternating-access mechanism, which involves a rocker-switch type movement of the two halves of the protein (Law et al., 2008). Salt-bridge formation and breakage are involved in the conformational changes of the protein during transport. Chloroplast secondary transporters belonging to various subclasses are described below and summarized in Table 14.3.

#### 14.5.2.1 Phosphate Transporters

Inorganic phosphate ( $P_i$ ) is an essential nutrient for plants and has both structural and regulatory roles, including modulation of photosynthesis. The concentration of  $P_i$  has to be maintained within relatively narrow limits, and this is achieved through metabolic recycling and transport across cellular membranes. Phosphate transporters are essential for chloroplasts since they control the  $P_i$  level in the stroma and the homeostasis required to initiate the Calvin cycle. Combination of experimental evidence and genome sequence analysis indicate that plants contain a wide variety of  $P_i$  transporter genes. So far, five distinct families of  $P_i$  transporters have been characterized, namely, PHT1 to PHT4 and pPT, each of them composed of multiple members (Rausch and Bucher, 2002; Knappe et al., 2003a; Guo et al., 2008a). Members of the PHT1 family are located in the plasma membrane and mediate  $P_i$  uptake into cells, whereas members of PHT2, PHT3, PHT4, and pPT families mediate translocation of  $P_i$  across organellar membranes. PHT2, pPT, and three out of the six PHT4 members (PHT4;3, PHT4;4 and PHT4;5) are chloroplast inner envelope proteins, whereas one PHT4 member is a thylakoid protein (Ruiz Pavón et al., 2008). PHT3 members are located to the inner mitochondrial membrane.

##### 14.5.2.1.1 Major Facilitators

PHT4 family is the most recently characterized PHT family (Guo et al., 2008a). It belongs to MFS and shares similarities with the mammalian SLC17/type I  $P_i$  transporters (T.C.#2.A.1.14.13). Therefore, PHT4 has been initially annotated as the anion transporter (ANTR) family. However, in contrast to SLC17, which has broad substrate specificity ( $P_i$ , organic anions and chloride), PHT4 members have been characterized in both yeast and *E. coli* as specific  $P_i$  transporters (Guo et al., 2008a; Ruiz Pavón et al., 2008). The difference between the two expression systems was in the nature of the cotransported ion since the first member (PHT4;1 *alias* ANTR1) has been characterized as a  $H^+$ -dependent  $P_i$  transporter in yeast and as a  $Na^+$ -dependent one in *E. coli*. Five of the six PHT4 members are targeted to plastids, and the sixth one to the Golgi (Guo et al., 2008a).

Among the plastid members, PHT4;2 (ANTR3) was exclusively found in the roots, whereas the other four are present in both leaves and roots but predominate in leaves, indicating a main function in the chloroplast. Two plastid members have been assigned a specific intra-chloroplast location, namely, PHT4;1 and PHT4;4 (ANTR2), localized to the thylakoid and envelope membrane, respectively (Ferro et al., 2003; Roth et al., 2005; Ruiz Pavón et al., 2008; Bräutigam et al., 2008). PHT4;1 has been shown to have a circadian-rhythm-regulated expression pattern (Guo et al., 2008b).

The function of none of the PHT4 members has been studied in *Arabidopsis* organelles, but it is expected that the direction of  $P_i$  transport is regulated either by the  $H^+$  or  $Na^+$  ions gradient. Whether these ions are cotransported with  $P_i$  by PHT4 members has not been demonstrated either. Most recently, site-directed mutagenesis of the AtANTR1 has revealed important residues for its transport activity (Ruiz Pavón, Karlsson et al., 2009). In plants, PHT4;1 is proposed to recirculate  $P_i$  from the lumen to the stroma during ATP transport and GTP signaling across the thylakoid membrane (Figure 14.3 and Section 14.4), whereas PHT4;4 has been proposed to supply stroma with cytosolic  $P_i$  (Guo et al., 2008a). Knockout mutants of PHT4;1 display reduced growth and high levels of photoprotection (Karlsson et al., 2010).

Combining proteomics and *in silico* approaches, a number of Na<sup>+</sup>-dependent putative transporters have been identified in the chloroplast inner envelope membranes, such as Na<sup>+</sup>Pi transporter, Na<sup>+</sup>/taurocholate transporters, Na<sup>+</sup>/H<sup>+</sup> antiporter, and Na<sup>+</sup>-ascorbate transporter (Ferro et al., 2003). Although their role in chloroplast is not clear, their homologues in bacteria or animal system are known to play a role in pH and Na<sup>+</sup> homeostasis.

#### 14.5.2.1.2 Drug/Metabolite Transporters

The plastid phosphate transport (pPT) family is part of the drug/metabolite transporter (DMT) superfamily (T.C. #2.A.7). The pPT family consists of 16 members in *Arabidopsis*, and among them, 10 are probably pseudogenes (Knappe et al., 2003a). pPTs function as antiporters using Pi and phosphorylated compounds as counter-substrates. The most prominent member of the pPT family in the chloroplast envelope is the triose-phosphate/phosphate (TPT) antiporter. It was the first plant transporter cloned and with the primary sequence determined (Flügge et al., 1989). TPT has the role to export dihydroxy-acetone-phosphate generated during Calvin cycle to the cytosol, where it is used for sucrose and cell wall biosynthesis. It has been reported to be a voltage-dependent ion channel permeable to anions as well as an antiporter (Flügge, 1999). A single gene for TPT is present in *Arabidopsis* and is expressed in photosynthetically active tissues. TPT provides a link between chloroplast and cytosolic metabolism. A moderate reduction in the TPT activity leads to drastic perturbations of the leaf metabolism, since the lack of TP export for cytosolic sucrose biosynthesis appears to be compensated by continuous accelerated starch turnover as well as by export of neutral sugars from the stroma throughout the day. The utilization of glucose 6-phosphate (generated from exported glucose) rather than TP for sucrose biosynthesis in the light bypasses the key regulatory step catalyzed by cytosolic fructose 1,6-bisphosphatase (Schneider et al., 2002). Interestingly, the *ape2* mutant, carrying an insertion in the TPT gene, displayed altered photoprotection during growth light conditions (Walters et al., 2003). Furthermore, overall reduced rates of electron transport, due to reduction in PSII photochemical efficiency, was observed in the *ape2* mutant, and therefore proposed that export of photosynthetic products from the chloroplast *via* TPT is crucial for the maintenance of high rates of photosynthetic electron transport.

Another well-characterized phosphate transporter is phosphoenolpyruvate/phosphate transporter (PPT), which imports phosphoenolpyruvate (PEP) from the cytosol and drives fatty acid synthesis and synthesis of compounds by the shikimate pathway (e.g., aromatic amino acids). A third phosphate transporter is glucose-6-phosphate/phosphate antiporter (GPT), present only in heterotrophic plastids. In *Arabidopsis*, two genes coding for PPTs exist, namely, AtPPT1 and AtPPT2. The phenotype of the *cuel* mutant, carrying an insertion in the PPT1 gene, revealed impaired leaf development. Obviously, in *Arabidopsis*, there are no known metabolic pathways or transporters that can compensate for the loss of AtPPT1, including AtPPT2. Despite a low sequence similarity, both PPTs are located in chloroplasts and possess similar substrate specificities. Expression of the two PPT genes is distinct in different tissues and cell types (Knappe et al., 2003b). The *AtPPT1* gene was found expressed in all organs examined, whereas *AtPPT2* transcripts could be detected only in leaves and flowers. Because a functional AtPPT1 is obviously crucial for correct leaf development, it is suggested that AtPPT1 is presumably involved in the synthesis of secondary metabolites that trigger leaf development (Knappe et al., 2003b).

#### 14.5.2.1.3 Inorganic Phosphate Transporters

PHT2;1 is a low-affinity transporter belonging to the Pi transporter family ((PiT; T.C. #2.A.20). It is expressed in both autotrophic and heterotrophic tissues, and its expression in leaves is up-regulated by light (Rausch et al., 2004). However, the use of inhibitors of photosynthesis did not interfere with the expression, indicating that it is not the photosynthesis *per se* that increases the expression. Moreover, modulation of its expression did not have any impact on the photosynthetic activity. Instead, it has been proposed that Pi import *via* PHT2;1 counterbalances the ADP/ATP exchange

mediated *via* the ADP/ATP translocator (see below), and thus maintain the Pi homeostasis in both green and non-green tissues.

#### 14.5.2.2 ATP/ADP Antiporters

Nucleotides are essential for living cells, since they are the building blocks for nucleic acids, drive energy-dependent processes, and play critical role in signal transduction. Moreover, they also serve as cofactors for various enzymes such as nicotinamide adenine dinucleotides used for redox reactions. Nucleotides are essential in all organelles and require specific transporters.

Two structurally and phylogenetically different types of intracellular adenine nucleotide transporters have been identified in chloroplasts, belonging to either the ATP/ADP antiporter (AAA) family or to the mitochondrial carrier family. The ATP/ADP antiporters (T.C. #2.A.12) import ATP in exchange for ADP, reside in the plastid inner envelope membrane of plants, and originate from the cytoplasmic membrane of parasite bacteria (Winkler and Neuhaus, 1999). The activity of a spinach plastidic ATP/ADP translocator was characterized already in 1969 by Heldt, as supplying the stroma with ATP from the cytosol during the dark period (Heldt, 1969). The responsible proteins were later identified in *Arabidopsis*, and named plastidic nucleotide translocators AtNTT1 and AtNTT2 (Kampfenkel et al., 1995; Möhlmann et al., 1998).

The activity of AtNTTs is important in several types of plastids, such as amyloplasts and chloroplasts, and their major physiological role is to supply plastids with ATP. *AtNTT1* transcript accumulates in leaf discs at high sugar concentrations, indicating that this gene belongs to a group of sugar up-regulated genes that reprogram chloroplasts into starch-storing plastids. Expression of *AtNTT2* is up-regulated in root tips and cotyledons of developing seedlings (Reiser et al., 2004). In adult plants, *AtNTT2* transcript is present in similar amounts in roots, leaves, stem, and flower tissues. Plants lacking a functional *AtNTT1* gene did not show altered root formation, chlorophyll synthesis, or seedling development, correlating with the higher expression of the *AtNTT2* gene in those tissues (Reiser et al., 2004). Instead, inactivation of the *AtNTT2* gene resulted in impaired formation of roots, most likely due to an inhibited rate of fatty acid synthesis in root plastids, which had been shown to be strictly dependent on the external supply of ATP. Furthermore, phenotypic analyses of *AtNTT2* knockout mutants indicated a reduced amount of thylakoid structures in the early developmental stage. The same authors have suggested that AtNTT2 is involved in supplying ATP for chlorophyll synthesis and the import of nuclear-encoded thylakoid proteins, and that alternative ATP transport pathways cannot compensate for this activity.

#### 14.5.2.3 Mitochondrial Carriers

The mitochondrial carriers (MC; T.C. #2.A.29) are proteins found only in eukaryotic cells. As the name indicates, the proteins belonging to this family were first recognized in the mitochondrial inner membrane. In addition to the mitochondrion, such carriers have also been found in the peroxisome, hydrogenosome, amyloplast, and chloroplast (Laloi, 1999; Thuswaldner et al., 2007; Palmieri et al., 2009). The *Arabidopsis* genome contains 58 membrane proteins belonging to MC family. The most studied MC members are the mitochondrial ADP/ATP carriers (AAC; T.C. #2.A.29.1.1). Like the members of the AAA family, AACs are antiporters, but the direction of transport is opposite, i.e., they import ADP into the matrix and export ATP to the cytosol through the intermembrane space. At least five AACs have been predicted in *Arabidopsis* by alignment with orthologous sequences, and some of them have been heterologously expressed and functionally characterized (Picault et al., 2004).

The activity of the first chloroplast MC member was reported in the spinach thylakoid membrane (Spetea et al., 2004), transporting ATP into the lumenal space. Searching for proteins responsible for this transthylakoid activity, one candidate in *Arabidopsis* was found and named thylakoid ATP/ADP carrier (TAAC) (Thuswaldner et al., 2007; Figure 14.3). The protein has been localized to the thylakoid membrane using immunogold labeling, Western blotting, and most recently using proteomics (Thuswaldner et al., 2007; Zybailov et al., 2008). A recombinant AtTAAC protein was

heterologously expressed in *E. coli* and found functionally inserted into the cytoplasmic membrane, allowing uptake studies to be performed in intact cells. Such experiments revealed an ATP/ADP exchange type of transport.

The *AtTAAC* gene is highly expressed in young photosynthetic organs, such as developing leaves, flower buds, and green siliques (Thuswaldner et al., 2007). Therefore, a role for TAAC in thylakoid biogenesis was proposed. TAAC expression is strongly up-regulated in leaves undergoing senescence or exposed to wounding, light stress, oxidative stress, salt stress, and desiccation, pointing to an additional role in supplying ATP for energy-dependent processes during thylakoid turnover. Detailed phenotypic analyses of two *taac* knockout mutants indicated a reduced ATP transthylakoid transport, better photoprotection during short-term high light stress, and increased sensitivity to prolonged high light stress as compared to the wild type (Thuswaldner et al., 2007; Yin et al., 2010). In the *taac* mutant, the PSII repair cycle is impaired, most likely not due to a blockage of D1 protein synthesis, but rather due to an inhibition of its degradation (see also Section 14.4).

The Brittle 1 (BT1) protein is an adenine nucleotide uniporter, in contrast to most MCs functioning as antiporters. It was found in potato, maize, and rice, and a putative homologue exists in *Arabidopsis* (Leroch et al., 2005). BT1 from *Zea mays* has been shown to transport ADP-glucose into endosperm plastids, and participate in starch biosynthesis (Kirchberg et al., 2007). The potato orthologue (StBT1) exports adenine nucleotides synthesized in plastids to the cytosol to be used in a variety of metabolic processes, and has a similar affinity for AMP, ADP, and ATP. As compared to TAAC, the expression of *StBT1* gene is similar in root, stem, leaf, and flower (with highest expression in flowers), indicating a general role in plant metabolism (Leroch et al., 2005).

Other two MC members in *Arabidopsis* have been characterized as NAD<sup>+</sup> carrier proteins, one in the chloroplast inner envelope membrane (AtNDT1) and one in mitochondria (AtNDT2) (Palmieri et al., 2009). Both carriers transport NAD<sup>+</sup> in counter-exchange with ADP or AMP, are highly expressed in metabolically active cells, and proposed to play a role in NAD<sup>+</sup>-dependent metabolic pathways and in the redox balance of chloroplasts and mitochondria.

#### 14.5.2.4 The Monovalent Cation:Proton Antiporter-2 (CPA2)

Na<sup>+</sup>/H<sup>+</sup> antiporters influence H<sup>+</sup> or Na<sup>+</sup> ion motive force across the membrane. Recently, such a protein (NhaS3) has been localized to the thylakoid membrane of *Synechocystis* sp. PCC 6803 (Tsunekawa et al., 2009). It belongs to the monovalent cation:proton antiporter-2 family (CPA2, T.C. #2.A.37). Its expression has been found regulated by the circadian rhythm, and it has been proposed to function as a putative uncoupler of the electrochemical proton gradient generated during photosynthesis. The closest *Arabidopsis* homologue of this cyanobacterial transporter is AtCXH23 reported to localize to the envelope (Song et al., 2004). RNA interference inhibition of its expression leads to morphological changes in the chloroplast. Plastids from *chx23* mutants had straight thylakoids but lacked grana lamellae. The *chx23* mutant leaves were pale yellow and had reduced chlorophyll content. The cytosolic pH in the leaves of the mutant was significantly higher than that in the wild type. *chx23* mutants displayed a high sensitivity to NaCl. CHX23 is a probable chloroplast Na<sup>+</sup>(K<sup>+</sup>)/H<sup>+</sup> exchanger important for pH homeostasis and chloroplast development and function.

Although most plants have been reported to have a low Na<sup>+</sup> content in chloroplast stroma, it is possible that sequestration of this ion into chloroplasts occurs as a partial detoxification mechanism in *Arabidopsis* or most other plants. Although a thylakoid Na<sup>+</sup>/H<sup>+</sup> antiporter has not been yet identified in *Arabidopsis*, such an activity is required, as judged, for example, on the presence of a Na<sup>+</sup>-dependent Pi transporter (Ruiz Pavón et al., 2008), supposed to eliminate Na<sup>+</sup> from the lumen. In addition, it has been reported that NaCl stress inactivates PSII oxygen-evolving complex as well as PSI electron transport in *Synechococcus* sp. PCC 7942. This effect is prevented by the addition of Na<sup>+</sup>-channel and water channel blockers, indicating that NaCl has both osmotic and ionic effects (Allakhverdiev et al., 2000).



### 14.5.3 PRIMARY TRANSPORTERS

Primary transporters (pumps, T.C. #3) directly use energy to transport molecules across a membrane. Most of the enzymes that perform this type of transport are transmembrane ATPases. A primary ATPase universal to all cellular life is the  $\text{Na}^+/\text{K}^+$  pump, which helps to maintain the cell potential. Other sources of energy for primary active transport are redox energy and photon energy (light). An example of primary active transport using redox energy is the mitochondrial electron transport chain, which uses the reduction energy of NADH to move protons across the inner mitochondrial membrane against their concentration gradient. An example of primary transport using light energy is the photosynthetic electron transport chain that uses the energy of photons to translocate  $\text{H}^+$  across the thylakoid membrane, also against their concentration gradient, and results in the production of reducing power in the form of NADPH. Chloroplast pumps belonging to various subclasses are described below and summarized in Table 14.4.

#### 14.5.3.1 ATP-Binding Cassette Transporters

ABC proteins (T.C. #3.A.1) represent one of the largest known families of proteins, and are present in bacteria, fungi, animals as well as plants (Martinoia et al., 2002). Most members are transporters that use the energy of ATP hydrolysis for driving translocation of various substrates. Characteristic for ABC transporters is their ability to use other nucleotides, mainly GTP, as a substitute for ATP, and the inhibition of their activity by vanadate. They participate in various processes, such as excretion of toxic substances, lipid translocation, conferring heavy-metal tolerance, antigen presentation, and exhibiting ion channel activity (Martinoia et al., 2002).

Among the 103 predicted ABC transporters in *Arabidopsis*, 14 were predicted as chloroplast proteins (Spetea and Thuswaldner, 2008). A few of them have been identified in chloroplast envelope membranes by proteomics (Kleffmann et al., 2004), but none of them has been characterized in terms of substrate and transport properties.

#### 14.5.3.2 $\text{H}^+$ -Translocating F-Type ATPases

The F-type ATPases ( $\text{F}_0\text{F}_1$ -ATPase; T.C. #3.A.2.1.1) generate ATP from ADP and phosphate, using the electrochemical proton gradient as a driving force. Thus,  $\text{F}_0\text{F}_1$  is an enzymatic machinery combining mechanical, electrical, and chemical aspects. The electrochemical motif force powers the electrical rotary motor  $\text{F}_0$ , driving in its turn the chemical motor  $\text{F}_1$  to produce ATP.  $\text{F}_0\text{F}_1$  can work in either direction, depending on which driving force dominates (unless is down-regulated for ATP hydrolysis, as in chloroplasts). The latest insights into the rotary mechanism of this machinery have been reviewed, revealing torque generation and an elastic mechanical-power transmission from  $\text{F}_0$  to  $\text{F}_1$  (Junge et al., 2009). The elastic power transmission between the two motors is proposed to smooth their cooperation without the need for fine tuning.

$\text{F}_0\text{F}_1$ -ATPases are present in both prokaryotic and eukaryotic organisms, localized in energy-transducing membranes, such as the chloroplast thylakoid membrane, the mitochondrial inner membrane, and the cytoplasmic membrane of some bacteria. The chloroplast ATP synthase ( $\text{CF}_0\text{F}_1$ -ATPase) is located in the thylakoid membrane, completing the energy transduction of the electron transport chain by synthesizing ATP (Figure 14.3 and Nelson and Ben-Shem, 2004). Six subunits of the chloroplast ATP synthase are chloroplast encoded, while only three subunits are nuclear-encoded proteins. The gamma subunit has been shown to be crucial for photoprotection, since its inactivation by T-DNA insertion mutagenesis destabilized the entire ATP synthase complex, and abolished the photophosphorylation reaction. Instead, a high level of proton accumulation was observed in the thylakoid lumen leading to swollen thylakoids and unusually high levels of NPQ of chlorophyll fluorescence (Bosco et al., 2004). Although there are no reports on mutagenesis of other major subunits of the ATP synthase subunits from *Arabidopsis*, it is expected that the effects would be very similar to those observed for the gamma subunit.



The activity of a chloroplast inner envelope  $H^+$ -ATPase has been also reported in the past (Berkowitz and Peters, 1993). It transports  $H^+$  from the stroma to the cytosol using the energy of ATP hydrolysis to maintain a pH gradient between stroma and cytosol, and thus optimize photosynthetic capacity. However, the protein components of the envelope  $H^+$ -ATPase have not yet been identified.

#### 14.5.3.3 P-Type ATPases

The P-type ATPase superfamily (P-ATPase, T.C. #3.A.3) consists of pumps transporting charged substrates, including  $H^+$ ,  $Na^+$ ,  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ ,  $Cd^{2+}$ ,  $Zn^{2+}$  ions, and phospholipids across membranes of both prokaryotes and eukaryotes. This superfamily includes a heavy-metal  $P_{1B}$ -ATPase (HMA) family that translocates cations out of the cytoplasm across biological membranes using energy from ATP hydrolysis. Among the 46 P-ATPases predicted in *Arabidopsis*, 3 have been so far identified and characterized in the chloroplast inner envelope membrane, namely, the  $Cu^{2+}$ -ATPase PAA1 (Shikanai et al., 2003), the  $Cu^{2+}$ ,  $Ca$ , and  $Zn^{2+}$ -ATPase HMA1 (Seigneurin-Berny et al., 2006; Moreno et al., 2008; Kim et al., 2009), and the autoinhibiting  $Ca^{2+}$ -ATPase ACA1 (Huang et al., 1993), and 1 in the thylakoid membrane, namely, the  $Cu^{2+}$ -ATPase PAA2 (Abdel-Ghany et al., 2005) (Figure 14.3). Unlike other plant P-ATPases, HMA1 appears to have a broad substrate specificity, explained by the observed difference in amino acids important for substrate specificity, and by the fact that evolutionarily it is more ancient than other members of the HMA family (Kim et al., 2009).

PAA1 and PAA2 transport copper into the chloroplast, and further across the thylakoid membrane, supplying the lumen with this essential cofactor, which plays a role in the photosynthetic electron transport through the plastocyanin (Shikanai et al., 2003; Abdel-Ghany et al., 2005). Copper is also a cofactor for the chloroplast Cu/Zn-superoxide dismutase (SOD), critical for the defense against many oxidative stress situations (Bertrand and Poirier, 2005; see also Chapter 26). Analysis of *paal* knockout mutants revealed deficiency in photosynthetic electron transport, likely due to impaired import of  $Cu^{2+}$  into the chloroplast resulting in insufficient formation of holoplastocyanin and lack of Cu/Zn-SOD in green tissues (Shikanai et al., 2003; Abdel-Ghany et al., 2005). The *paal2* mutant had a similar, though not as severe, phenotype. The double *paal1-2* mutant was seedling lethal (Abdel-Ghany et al., 2005). The *hma1* knockout plants were shown to be photosensitive under high light, consistent with the decrease in the chloroplast copper content and reduction in the total chloroplast SOD activity (Seigneurin-Berny et al., 2006). Furthermore, the ATPase activity of HMA1 was shown to be lower than that of PAA1, though they were not able to replace each other. These results suggest that, though functional in the same (envelope) membrane, PAA1 is the main  $Cu^{2+}$ -ATPase, whereas HMA1 may play a different role in chloroplasts. Most recently, it has been shown that when grown in the presence of high  $Zn^{2+}$  concentrations, the *hma1* mutant displays shoot weight and chlorophyll content that are below 40% of the wild type. The explanation of this phenotype is that the mutant accumulates more  $Zn^{2+}$  than the wild type in the shoot, and therefore it has been proposed that HMA1 protects the chloroplast against toxicity of  $Zn^{2+}$  by exporting it to the cytoplasm. No phenotype related to  $Cu^{2+}$ ,  $Ca^{2+}$ , or heavy metal has been found for HMA1 to support the  $Cu^{2+}$  and  $Ca^{2+}$ -transporting activity. The light-sensitive phenotype observed by Seigneurin-Berny et al. (2006) was explained by the fact that excess  $Zn^{2+}$  could replace  $Cu^{2+}$  in SOD.

#### 14.5.3.4 Light-Absorption-Driven Transporters

Light-absorption-driven transporters (T.C. #3.E) are transport systems that utilize light energy to drive transport of a solute, and are divided in two subclasses: the ion-translocating rhodopsin family (T.C. #3.E.1) and the photosynthetic reaction center (PRC, T.C. #3.E.2). There are two types of PRCs in the chloroplast thylakoid membrane, associated with PSII and PSI. They use the energy of absorbed photons to translocate electrons across the membrane. Similarly, bacterial RCs together with the cyt *b<sub>6</sub>f* complex mediate the conversion of light into electrochemical energy by transmembrane electron and  $H^+$  transport during photosynthesis. In this process, electron transfer to quinone

is coupled to proton transfer. Both PSI and PSII of plants and cyanobacteria belong to the PRC superfamily, but they are more complex than the purple bacterial members. The reaction center subunits of PSII are D1 and D2, which bind/contain the redox components in the electron transfer chain. D1 is the most important subunit for PSII since it binds most of the active components, is most prone to photodamage, and requires efficient repair in order that the organism survives in its natural or stressful light environment (see Section 14.2).

#### 14.5.4 INCOMPLETELY CHARACTERIZED TRANSPORTERS

Transport protein families of unknown classification (T.C. #9) are grouped in this category, and will hopefully be classified elsewhere when the transport mode and/or energy coupling mechanism will be characterized. These families include at least one member for which a transport function has been established, but neither the mode of transport nor the energy coupling mechanism is still known. To this category belong the chloroplast metal ion transporters described in Section 14.5.4.1 and summarized in Table 14.4.

##### 14.5.4.1 Metal Ion Transporters

Transition metal transporters maintain metal concentration within physiological limits in all plant organs where enzymes and proteins functionally associated with metals ( $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ni}^{2+}$ ) are present. Particularly, the photosynthetic machinery in plants is abundant in metal-containing proteins, with  $\text{Fe}^{2+}$  being the dominant type in PSII, PSI, *cyt  $b_6f$* , and ferredoxin followed by  $\text{Mn}^{2+}$  in the oxygen-evolving complex of PSII, and  $\text{Cu}^{2+}$  in plastocyanin (Merchant and Dreyfuss, 1998). The reason why the three metal ions play a vital role in photosynthetic electron transport in chloroplasts resides in their potential for valency changes. There are many different known transition metal transport families (Krämer et al., 2007). It seems that there are specific characteristics for transporters of essential metals and for toxic metals from those for other small molecules or ions. For example, transition metal ions present in aqueous solution form in the cytoplasm can disrupt cellular functions. Based on metal-binding affinities of apo-metalloproteins, it has been estimated that the cytoplasmic concentrations can correspond to less than one free metal ion per cell. In other words, there is no persistent pool of free transition metal ions, from which metal transporters can bind metal ions. Instead, it is thought that these transporters acquire their metal substrates through interaction with metallochaperone shuttle proteins (O'Halloran and Culotta, 2000). Despite these essential functions for metal ions in photosynthesis, very little is known about metal transport proteins in chloroplasts. To date, the only chloroplast proteins demonstrated to be involved in metal ion transport are the  $\text{Mg}^{2+}$  transport protein MRS2-11, the  $\text{Cu}^{2+}$ -transporting P-type ATPases PAA1 and PAA2, and the heavy-metal ATPase HMA1, discussed in Sections 14.5.1.4 and 14.5.3.3.

Direct measurements of iron transport on isolated pea (*Pisum sativum*) chloroplasts have shown that iron is transported as  $\text{Fe}^{2+}$  ion across the inner envelope (Shingles et al., 2001, 2002). The putative metal uptake protein has been proposed to mediate  $\text{Fe}^{2+}/\text{H}^{+}$ -uniport and be able to transport  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Mn}^{2+}$  ions as well. Recently, the responsible protein has been identified in the inner envelope of *Arabidopsis* chloroplasts and named Permease In Chloroplasts 1 (PIC1) (Duy et al., 2007b). PIC1 has a cyanobacterial origin, and most likely functions as an iron permease in the chloroplast envelope. The phenotype of *pic1* knockout mutants resembled that of iron-deficient chloroplasts, i.e., chlorosis, displayed an altered organization of leaf mesophyll cells, and severe defects in chloroplast and thylakoid development. Furthermore, the expression of photosynthetic and Fe-S cluster genes is down-regulated in the *pic1* mutants. Instead, the ferritin clusters accumulate in the mutant plastids and many stress-related genes are up-regulated, indicating an iron-overload reaction in the cytosol and impaired metal homeostasis at the cellular level. Both PIC1 and its cyanobacterial homolog (slr11656) have been found to perform iron uptake and to complement the growth of an iron-uptake-defective yeast mutant, indicating that the protein is involved in iron transport and homeostasis in chloroplasts.

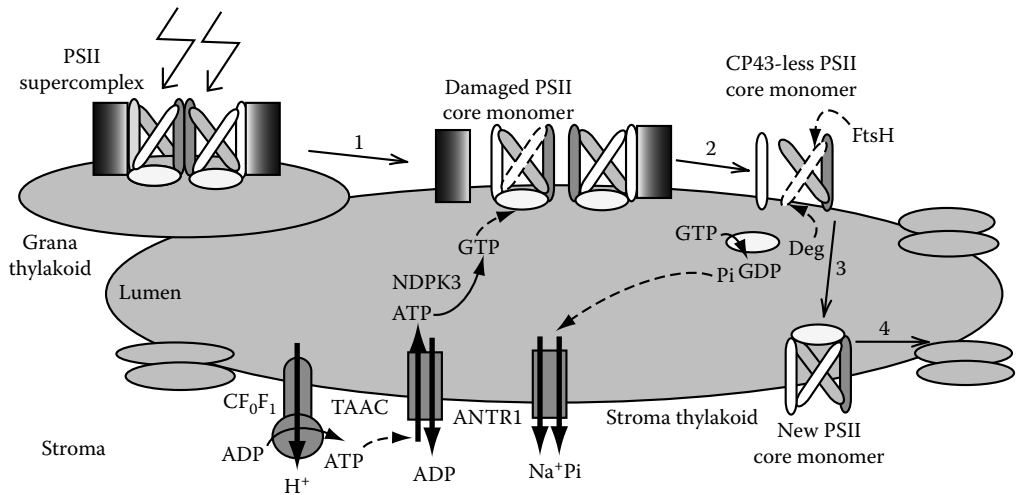
PIC1 has also been known as TIC21, since it has been found to be involved in a 1 MDa translocation complex during protein import across the chloroplast inner envelope membrane (Teng et al., 2006; Kikuchi et al., 2009). The 1 MDa translocation complex is neither the Toc complex, to which preproteins initially bind, nor the Tic110-containing complex, which should mediate a later translocation step on the stromal side, but functions in between the Toc- and the Tic110-containing complexes. Based on phenotypic analyses of *tic21* alias *pic1* mutants, it has been shown that TIC21 is not central for the import, but most likely a loosely associated component. Moreover, the phenotype of the mutant was shown to resemble that of other albino mutants, and thus not directly related to iron homeostasis.

Another transporter with potential role in iron homeostasis is RTS3. This protein has been initially predicted as a chloroplast inner envelope protein and as an antibiotic (aminoglycoside) transporter (Aufsatz et al., 2009). The experimental proof for its location and function was brought by Conte et al. (2009). Therefore, RTS3 has been renamed to multiple antibiotic resistance 1 (MAR1). Based on the identified function and localization to the plant chloroplast, it is unlikely that evolutionary pressure has selected for mean of entry for toxic antibiotics into this organelle. The transport of antibiotics is most likely an opportunistic effect, explained by the fact that the prokaryotic and the chloroplast machineries are very similar and that the bacterial plasma membrane and the chloroplast (inner) envelope membrane contain similar types of transporters. The natural function of MAR1 is unknown, however, based on its similarity to ferroportin (T.C. #9.A.23), a function in  $\text{Fe}^{2+}/\text{H}^{+}$  antiport, and a role in cellular iron homeostasis have been proposed. The mutants overexpressing MAR1 show chlorosis, a common symptom of iron deficiency in plants since iron is essential for chlorophyll biosynthesis. Since this phenotype is rescued by iron feeding, MAR1 may play a role in iron chelation, storage, and/or sequestration. Thus, MAR1 may be transporting iron or a molecule (e.g., nicotinamide) involved in iron homeostasis into the chloroplast. MAR1 may not be able to distinguish between this molecule and aminoglycosides.

## 14.6 MODEL FOR THE ROLE OF THYLAKOID TRANSPORTERS IN LIGHT STRESS

The water-oxidizing PSII complex has attracted a special attention, since its reaction center D1 subunit is degraded and replaced much faster than the other subunits under excess and even optimal light conditions (see Section 14.2). Thus, D1 protein turnover is the major event in the repair cycle of the PSII complex, and occurs subsequently to the inactivation of PSII electron transport. D1 protein degradation is a multistep proteolytic event, requiring GTP and ATP, and performed most likely by Deg and FtsH proteases (Spetea et al., 1999; Lindahl et al., 2000; Haussühl et al., 2001; Silva et al., 2003; Kapri-Pardes et al., 2007). The PSII repair cycle is regulated by reversible phosphorylation of several core subunits, including D1 (Tikkanen et al., 2008). The repair process step-by-step could be described as follows: phosphorylation and damage of PSII dimers by high light in the grana thylakoids, monomerization and migration to the stroma thylakoids, dephosphorylation and dissociation of the CP43 subunit from the PSII core monomer, degradation of the D1 subunit; *de novo* synthesis of a new D1 protein; co-translational insertion of the D1 and concomitant assembly of the PSII monomer; migration back to the grana thylakoids, dimerization, and reactivation of the oxygen-evolving complex (Aro et al., 1993; Aro et al., 2005). The major steps are shown in Figure 14.4, where, for clarity, the phosphorylation/dephosphorylation events have been omitted.

To perform this multistep cycle in an efficient way for the plant to cope with the ambient light conditions, a large number of regulatory and transport proteins are required. Most of them are still unknown despite their crucial importance, in the frame of molecular agriculture, to obtain varieties with improved crop production and tolerance to stress. *Arabidopsis* has been an excellent model plant to identify such components with the help of available genomic information and a wide variety of genetic tools (see Section 14.3). Using a functional genomics approach, four thylakoid transporters have been identified and characterized at molecular level (see Figure 14.3). Special emphasis is



**FIGURE 14.4** Schematic model of the sequence of events during damage and repair of photosystem II (PSII) complex exposed to high light stress. Step 1: PSII inactivation, D1 protein damage, PSII monomerization, and migration to the stroma lamellae; Step 2: dissociation of CP43 and PsbO subunits from the PSII monomer, degradation of the D1 protein by Deg and FtsH proteases; Step 3: *de novo* synthesis and co-translational insertion of a new D1 protein, reassembly of the PSII monomer; Step 4: migration to the grana lamellae, dimerization, and activation. Based on very recent reports (see text for details), transthylakoid ATP transport and GTP signaling are proposed to play critical roles during PSII repair cycle as follows: ATP synthase supplies ATP in the stroma, which is translocated by the thylakoid ATP/ADP carrier (TAAC) into the lumen in exchange for ADP. The luminal nucleoside diphosphate kinase 3 (NDPK3) converts ATP to GTP, which is bound and hydrolyzed by the PsbO subunit extrinsic luminal subunit of PSII, leading to its dissociation and subsequent PSII disassembly (Step 2) in a highly controlled manner. The resulting phosphate (Pi) is exported back to the stroma by the Na<sup>+</sup>-dependent Pi transporter (ANTR1).

given in this section to TAAC and ANTR1, since both may play a role in PSII repair and photo-protection. Due to their transport activity, they may provide the link between ATP synthesis on the stromal side of the thylakoid membrane and nucleotide-dependent reactions in the luminal space.

A model for their function during PSII repair cycle is presented in Figure 14.4, and explained below. The ATP translocated by TAAC across the thylakoid membrane is converted to GTP by the luminal nucleoside diphosphate kinase 3 (NDPK3). GTP is, then, bound and hydrolyzed by the PsbO luminal extrinsic subunit of the PSII complex (Spetea et al., 2004; Lundin et al., 2007a). Between the two PsbO isoforms in *Arabidopsis*, it has recently been reported that PsbO2 plays an essential role in D1 turnover during high light stress and has a higher GTPase activity than PsbO1 (Lundin et al., 2007b, 2008; Allahverdiyeva et al., 2009). The precise mechanism of PsbO2-mediated PSII repair is not yet known. Nevertheless, GTP was previously reported to be required for efficient proteolytic removal of the D1 protein during repair of photoinactivated PSII (Spetea et al., 1999). Furthermore, it has been proposed that the PsbO2-type of PSII complexes undergo a more efficient repair due to the PsbO2-mediated GTPase activity that induces PsbO2 release from the complex, thus facilitating the next steps in the repair process, namely, dissociation of the CP43 subunit and proteolysis of the D1 subunit (Lundin et al., 2007b, 2008). The Na<sup>+</sup>-dependent Pi transporter ANTR1 is proposed to play a role in exporting back to the stroma Pi generated during nucleotide metabolism including GTP hydrolysis (Ruiz Pavón et al., 2008).

In addition to the role in PSII repair, both transporters appear to influence the thermal photo-protection during high light stress (Yin et al., 2010; Karlsson et al., 2010). At least in the TAAC case, this effect is attributed to the fact that its transport activity is driven by the H<sup>+</sup> gradient across the thylakoid membrane (Thuswaldner et al., 2007). It is well known that adenine nucleotide exchanged by AAC across the mitochondrial membrane is electrogenic (ATP<sup>4-</sup>/ADP<sup>3-</sup>) since only

the unprotonated species  $\text{ATP}^+/\text{ADP}^{3-}$  are transported independently of pH in the range 6.2–7.4 (Gropp et al., 1999). It is likely that this is also valid for TAAC-catalyzed transport taking into account the similar structure and antiport mechanism for AAC and TAAC (Thuswaldner et al., 2007). More recent studies have shown that ionic strength of the medium can broaden the pH range of unprotonated species toward pH 5 (De Stefano et al., 2006), which is relevant for the thylakoid lumen.

The transport activity of ANTR1 has been shown to be dependent on electrochemical  $\text{Na}^+$  gradient, when assessed across the bacterial membrane (Ruiz Pavón et al., 2008). Furthermore, based on the determined kinetics parameters, a stoichiometry of  $>1$  for  $\text{Na}^+:\text{Pi}$  for ANTR1-mediated transport has been suggested. There are reports indicating an electrogenic transport for other  $\text{Pi}$  transporters (Bacconi et al., 2005), but not specifically for the SLC17 family, to which ANTR1 belongs. Nevertheless, the observation that mutants lacking this protein display higher levels of NPQ than the wild type (Karlsson et al., 2010) points out to a possible electrogenic mechanism for the thylakoid  $\text{Pi}$  transporter as well. Thus, it appears that to maintain steady and balanced electrochemical gradient across the thylakoid membrane, electrogenic transporters may modulate the activity of the xanthophyll cycle (see Chapter 16). To summarize, the thylakoid transporters may have multiple roles during light stress, which make them very important targets for plant breeding.

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# 15 Photosynthetic Pigment Apparatus in Northern Plants

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## 15.1 INTRODUCTION

In nature, plants frequently experience a wide range of stresses. An investigation of functional plasticity is necessary to understand responses to environmental conditions, the distribution of the species, and to predict the dynamics of vegetation under the global changes of climate. The indices of photosynthetic apparatus, such as the composition, the content, and the ratio of pigments are considered to be the most informative to characterize the functional plant state (Lubimenko 1963, Zalenskii 1977, Maslova and Popova 1993, Pyankov and Mokronosov 1993, Bazzaz 1996, Dymova and Golovko 2007, Golovko et al. 2007).

Chlorophylls and carotenoids are required for photosynthesis. Chlorophylls are necessary for the capture of light energy and as primary electron donors. Carotenoids play crucial roles in both light harvesting and energy dissipation for the protection of photosynthetic structures. So far, the spectral characteristics and the biosynthesis of photosynthetic pigments have been studied, the concept of the antenna complex and the reaction centers has been developed, and the fundamental mechanisms of photosynthesis have been revealed. At the same time, the diversity and ecological conditions of habitation of plant species make the investigation of the pigments' role in the stability and regulation of the photosynthetic apparatus activity the topical problem. A qualitative content and a quantitative composition of pigments and their ratio changes are the important and responsive characteristics of the physiological state of plants and their photosynthetic apparatus. However, current information about the pigment system of plants from different botanical and geographical zones is rare and contradictory (Lukyanova et al. 1986, Popova et al. 1989, Kornushenko and Solovjova 1992, Maslova and Popova 1993, Golovko et al. 2007).

Northeast European Russia is a unique region in continental Europe. Here, plant growth is restricted by lack of warmth, short vegetative period, and poor soils. Photosynthetic apparatus, including pigment complex, prove additional stress in these severe conditions.

We advance the idea of increasing the role of pigments in tolerance and in the productivity of photosynthesis of plants in the taiga cold climate environments. The results presented in this chapter provide proof for this idea.

15.2 PHOTOSYNTHETIC PIGMENT CONTENTS IN NORTHERN PLANTS

We studied the pigment complex of more than 100 plant species inhabiting three different sites in the European North-East of Russia: (1) the Subpolar Ural Mountains (65°22' N, 60°46' E), (2) the South Tyman (62°45' N, 55°49' E), and (3) the Middle Vychegda basin (61°38' N, 50°43' E).

The main climatic traits of these regions are shown in Table 15.1. The Subpolar Ural Mountains (the extremely north taiga subzone) is characterized by the most severe climate and a short vegetative period. The climate in the Middle Vychegda (the middle taiga subzone) is warmer, but the growing season in this region does not exceed 100–110 days. The mean annual air temperature only slightly exceeds 1°C. Though the region of the South Tyman belongs to the middle taiga subzone, it differs from the Middle Vychegda by the temperature regime and the duration of the vegetative period.

The list of examined plant species in each region is shown in Table 15.2. All 121 plant species have been investigated and most of them are boreal herbaceous plants and reflect the structure of the floristic complexes in the studied regions.

Leaf samples for pigment analyses were collected from 20 to 30 plants of each species from the beginning–middle of July (2004–2008). Analyses were carried out on mature healthy current-year leaves (herbs) or current-year shoots (*Empetrum nigrum* and *E. hermaphroditum*, *Lycopodium clavatum* and *L. annotinum*, *Diphasium complanatum*). The second-year needles were collected from evergreen conifers (*Abies sibirica*, *Juniperus communis*, *Picea obovata*, *Pinus sibirica*). The majority of herbaceous plants flowered during the sampling.

Leaf chlorophyll (Chl) and carotenoid (Car) contents were measured by UV-1700 spectrophotometer (“Shimadzu”, Japan) in acetone extracts at 662 (Chl *a*), 644 (Chl *b*), and 470 nm (total Car). The chlorophyll portion in light-harvesting chlorophyll (LHC) (LHC-Chl) was calculated, assuming that the total Chl *b* was located in LHC and the Chl *a/b* ratio in this complex was equal to 1.2 (Lichtenthaler 1987). Separations and quantifications of Car were done by reversed-phase high-performance liquid chromatography (HPLC) according to Gilmore and Yamamoto (1991).

15.2.1 PIGMENT COMPLEX OF THE SUBPOLAR URAL MOUNTAINS PLANTS

The differences in the accumulation of photosynthetic pigments were revealed between species (Figure 15.1). The concentration of Chl and Car varied in the ranges of 1.5–14 and 0.5–5 mg/g dry weight (DW), respectively. The legume plants (*Astragalus norvegicus*, *A. frigidus*, *Hedysarum arcticum*)

TABLE 15.1  
Mean Climatic Traits of Different Places in the European North-East

| Traits                                                   | Subpolar Ural Mountains | The South Tyman | The Middle Vychegda Basin |
|----------------------------------------------------------|-------------------------|-----------------|---------------------------|
| Mean annual air temperature, °C                          | −4.8                    | −1.5            | +1                        |
| Mean July air temperature, °C                            | +13                     | +15             | +17                       |
| Total temperature above +5°C                             | 1070                    | 1550            | 1800                      |
| The duration of growing season (above +5°C), days        | 105–110                 | 133             | 150                       |
| The duration of active growth period (above +10°C), days | 60–70                   | 80–90           | 100–110                   |
| Mean annual precipitation, mm                            | 600–685                 | 660–750         | 650–765                   |

TABLE 15.2

## List of the Species from Different Places in the European North-East

| No. | Subpolar Ural Mountains                                  | The South Tyman                                              | The Middle Taiga Subzone                                  |
|-----|----------------------------------------------------------|--------------------------------------------------------------|-----------------------------------------------------------|
| 1   | 2                                                        | 3                                                            | 4                                                         |
| 1   | <i>Achillea nigrescens</i> (E. Mey) Rydb<br>(H, B)       | <i>Aconitum septentrionale</i> Koelle.<br>(H, B)             | <i>Abies sibirica</i> Ledeb.<br>(T, B)                    |
| 2   | <i>Alchemilla murbeckiana</i> Buser<br>(H, B)            | <i>Antennaria dioica</i> (L.) Gaertn.<br>(H, B)              | <i>Achillea millefolium</i> L.<br>(H, B)                  |
| 3   | <i>Amoria repens</i> (L.) C. Presl<br>(H, B)             | <i>Aster sibiricus</i> L.<br>(H, A)                          | <i>Aconitum septentrionale</i> Koelle<br>(H, B)           |
| 4   | <i>Arctous alpina</i> (L.) Niedz.<br>(SH, A + AA)        | <i>Astragalus danicus</i> Retz.<br>(H, P)                    | <i>Alchemilla</i> sp.<br>(H, P)                           |
| 5   | <i>Artemisia tilesii</i> Ledeb.<br>(H, A + AA)           | <i>Calamagrostis epigeios</i> (L.) Roth<br>(H, B)            | <i>Alisma plantago-aquatica</i> L.<br>(H, B)              |
| 6   | <i>Astragalus frigidus</i> (L.) A. Gray<br>(H, A)        | <i>Caltha palustris</i> L.<br>(H, B)                         | <i>Antennaria dioica</i> (L.) Gaertn.<br>(H, B)           |
| 7   | <i>Astragalus norvegicus</i> Grauer<br>(H, A + AA)       | <i>Cortusa matthioli</i> L.<br>(H, B)                        | <i>Bistorta major</i> S.F. Gray<br>(H, B)                 |
| 8   | <i>Atragene sibirica</i> L.<br>(SH, B)                   | <i>Cotoneaster melanocarpa</i> Lodd.<br>(SH, B)              | <i>Bromopsis inermis</i> Leyss.<br>(H, B)                 |
| 9   | <i>Betula nana</i> L.<br>(SH, A)                         | <i>Crepis sibirica</i> L.<br>(H, B)                          | <i>Butomus umbellatus</i> L.<br>(H, B)                    |
| 10  | <i>Bartsia alpina</i> L.<br>(H, A + AA)                  | <i>Cypripedium calceolus</i> L.<br>(SH, B)                   | <i>Calla palustris</i> L.<br>(H, B)                       |
| 11  | <i>Calamagrostis purpurea</i><br>(Trin.) Trin.<br>(H, B) | <i>Dactylorhiza fuchsii</i> (Druce)<br>Soo<br>(H, B)         | <i>Chenopodium album</i> L.<br><br>(H, B)                 |
| 12  | <i>Carex aquatilis</i> Wahlenb.<br><br>(H, B)            | <i>Dendranthema zawadskii</i> (Herbich)<br>Tzvel.<br>(H, R)  | <i>Comarum palustre</i> L.<br><br>(H, B)                  |
| 13  | <i>Cystopteris dickieana</i> R. Sim.<br><br>(H, A + AA)  | <i>Diphasium complanatum</i> (L.) Rothm.<br>(C-M, B)         | <i>Deschampsia cespitosa</i> (L.)<br>Beauv<br>(H, B)      |
| 14  | <i>Diapensia lapponica</i> L.<br>(SH, A + AA)            | <i>Dryas octopetala</i> L.<br>(SH, AA)                       | <i>Dryopteris filix-mass</i> (L.) Schott.<br>(F, B)       |
| 15  | <i>Empetrum hermaphroditum</i><br>(Lange)<br>(SH, A)     | <i>Dryopteris filix-mas</i> (L.) Schott.<br>(F, B)           | <i>Elytrigia repens</i> (L.) Nevski<br><br>(H, B)         |
| 16  | <i>Hedysarum arcticum</i> B. Fedtsh.<br>(H, A + AA)      | <i>Equisetum palustre</i> L.<br>(H, B)                       | <i>Empetrum nigrum</i> L.<br>(SH, A)                      |
| 17  | <i>Larix sibirica</i> Ledeb.<br>(T, B)                   | <i>Epipactis atrorubens</i> (Hoffm.) Besser.<br>(H, B)       | <i>Filipendula ulmaria</i> (L.) Maxim<br>(H, B)           |
| 18  | <i>Ledum decumbens</i> L.<br>(H, A)                      | <i>Geum rivale</i> L.<br>(H, B)                              | <i>Galium boreale</i> L.<br>(H, B)                        |
| 19  | <i>Lycopodium clavatum</i> L.<br>(H, A)                  | <i>Gymnadenia conopsea</i> (L.) R.Br.<br>(H, B)              | <i>Gymnocarpium dryopteris</i> (L.)<br>(H, B)             |
| 20  | <i>Lycopodium annotinum</i> L.<br><br>(H, B)             | <i>Gymnocarpium robertianum</i> (Hoffm.)<br>Newm.<br>(SH, B) | <i>Hylotelephium triphyllum</i> (Haw.)<br>Holub<br>(H, B) |

(continued)

TABLE 15.2 (continued)

## List of the Species from Different Places in the European North-East

| No. | Subpolar Ural Mountains                                         | The South Tyman                                         | The Middle Taiga Subzone                                   |
|-----|-----------------------------------------------------------------|---------------------------------------------------------|------------------------------------------------------------|
| 1   | 2                                                               | 3                                                       | 4                                                          |
| 21  | <i>Pedicularis verticillata</i> L.<br>(H, A + AA)               | <i>Juniperus communis</i> L.<br>(SH, B)                 | <i>Hypericum maculatum</i> Crantz<br>(H, B)                |
| 22  | <i>Pentaphylloides fruticosa</i> (L.)<br>O. Schwarz.<br>(SH, B) | <i>Lathyrus pratensis</i> L.<br>(H, B)                  | <i>Juniperus communis</i> L.<br>(SH, B)                    |
| 23  | <i>Phyllodoce caerulea</i> (L.) Bab.<br>(SH, A + AA)            | <i>Lathyrus vernus</i> (L.) Bernh.<br>(H, B)            | <i>Ledum palustre</i> L.<br>(SH, B)                        |
| 24  | <i>Pyrola rotundifolia</i> L.<br>(H, B)                         | <i>Lycopodium annotinum</i> L.<br>(C-M, B)              | <i>Leontodon autumnalis</i> L.<br>(T, B)                   |
| 25  | <i>Rhodiola rosea</i> L.<br>(H, A)                              | <i>Melica nutans</i> L.<br>(H, B)                       | <i>Lycopodium clavatum</i> L.<br>(C-M, B)                  |
| 26  | <i>Rosa acicularis</i> Lindley.<br>(SH, B)                      | <i>Paeonia anomala</i> L.<br>(H, B)                     | <i>Maianthemum bifolium</i> (L.)<br>F.W. Schmidt<br>(H, B) |
| 27  | <i>Rubus chamaemorus</i> L.<br>(H, A)                           | <i>Paris quadrifolia</i> L.<br>(H, B)                   | <i>Oxalis acetosella</i> L.<br>(H, B)                      |
| 28  | <i>Salix dasyclados</i> Wimm.<br>(T, B)                         | <i>Parnassia palustris</i> L.<br>(H, B)                 | <i>Paris quadrifolia</i> L.<br>(H, B)                      |
| 29  | <i>Salix nummularia</i> L.<br>(SH, A + AA)                      | <i>Pedicularis verticillata</i> L.<br>(H, AA)           | <i>Petasites spurius</i> (Retz.)<br>(H, A)                 |
| 30  | <i>Salix reticulata</i> L.<br>(SH, A + AA)                      | <i>Petasites radiatus</i> (J.F. Gmel.) Holub.<br>(H, A) | <i>Pimpinella saxifraga</i> L.<br>(H, B)                   |
| 31  | <i>Tanacetum bipinnatum</i> (L.) Sch.<br>Bip.<br>(H, A)         | <i>Pinguicula vulgaris</i> L.<br>(H, A)                 | <i>Picea obovata</i> Ledeb<br>(T, B)                       |
| 32  | <i>Vaccinium myrtillus</i> L.<br>(SH, B)                        | <i>Pinus sibirica</i> Du Tour<br>(T, B)                 | <i>Pinus sibirica</i> Du Tour<br>(T, B)                    |
| 33  | <i>Vaccinium uliginosum</i> L.<br>(SH, B)                       | <i>Plantago media</i> L.<br>(H, B)                      | <i>Plantago major</i> L.<br>(T, B)                         |
| 34  | <i>Valeriana wolgensis</i> Kazak.<br>(H, B)                     | <i>Polygonum viviparum</i> L.<br>(H, AA)                | <i>Polygonum aviculare</i> L.<br>(H, B)                    |
| 35  | <i>Woodsia glabella</i> R.Br.<br>(H, A + AA)                    | <i>Pyrola rotundifolia</i> L.<br>(H, B)                 | <i>Pyrola rotundifolia</i> L.<br>(H, B)                    |
| 36  |                                                                 | <i>Sanguisorba officinalis</i> L.<br>(H, B)             | <i>Rorippa amphibia</i> (L.) Bess<br>(H, B)                |
| 37  |                                                                 | <i>Saussurea alpina</i> (L.) DC.<br>(H, AA)             | <i>Rosa majalis</i> Herrm.<br>(SH, B)                      |
| 38  |                                                                 | <i>Saxifraga hirculus</i> L.<br>(H, A)                  | <i>Rubus saxatilis</i> L.<br>(H, B)                        |
| 39  |                                                                 | <i>Thymus talijevii</i> Klok. et Shost.<br>(SH, B)      | <i>Rubus chamaemorus</i> L.<br>(H, A)                      |
| 40  |                                                                 | <i>Tofieldia pusilla</i> L.<br>(H, AA)                  | <i>Taraxacum officinale</i> Wigg.<br>(H, B)                |
| 41  |                                                                 | <i>Tussilago farfara</i> L.<br>(H, B)                   | <i>Thalictrum simplex</i> L.<br>(H, B)                     |
| 42  |                                                                 | <i>Vaccinium uliginosum</i> L.<br>(SH, A)               | <i>Trifolium medium</i> L.<br>(H, B)                       |

TABLE 15.2 (continued)  
List of the Species from Different Places in the European North-East

| No. | Subpolar Ural Mountains | The South Tyman                             | The Middle Taiga Subzone                   |
|-----|-------------------------|---------------------------------------------|--------------------------------------------|
| 1   | 2                       | 3                                           | 4                                          |
| 43  |                         | <i>Valeriana capitata</i> Pallas.<br>(H, B) | <i>Trifolium pratense</i> L.<br>(H, B)     |
| 44  |                         | <i>Valeriana wolgensis</i> Kazak.<br>(H, B) | <i>Veronica longifolia</i> L.<br>(H, B)    |
| 45  |                         | <i>Vicia cracca</i> L.<br>(H, B)            | <i>Vaccinium myrtillus</i> L.<br>(SH, B)   |
| 46  |                         | <i>Vicia sylvatica</i> L.<br>(H, B)         | <i>Vaccinium uliginosum</i> L.<br>(SH, A)  |
| 47  |                         | <i>Woodsia glabella</i> R.Br.<br>(SH, AA)   | <i>Vaccinium vitis-idaea</i> L.<br>(SH, A) |

Notes: Life-form groups: H—herb, T—tree, SH—shrub; C-M—club-moss; latitudinal groups: A—arctic, AA—arctic and alpine, B—boreal species; P—prairie, R—rocky species. Latin names of species given by Cherepanov (1995). Species presented with note of its life form and geographical status.

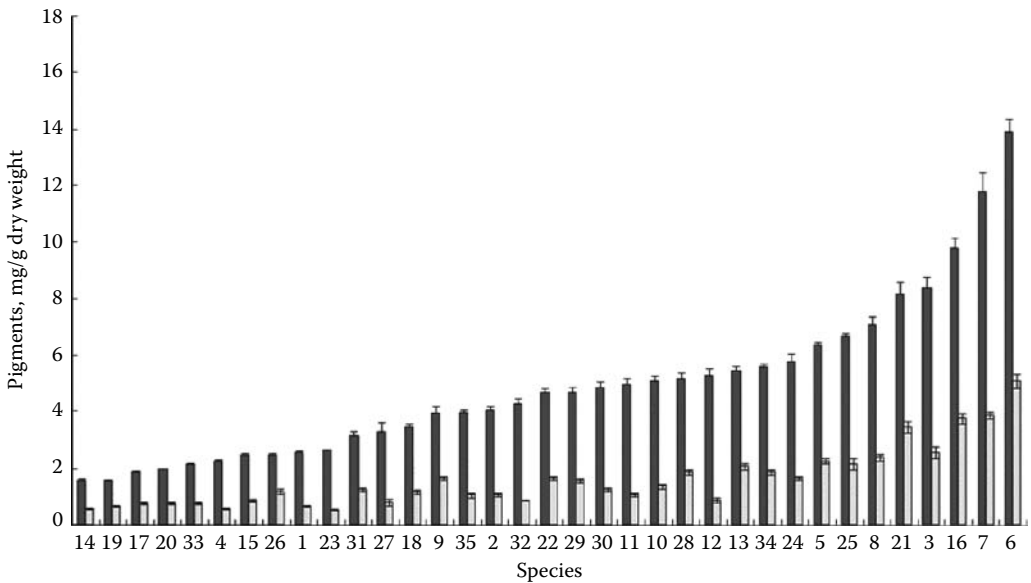
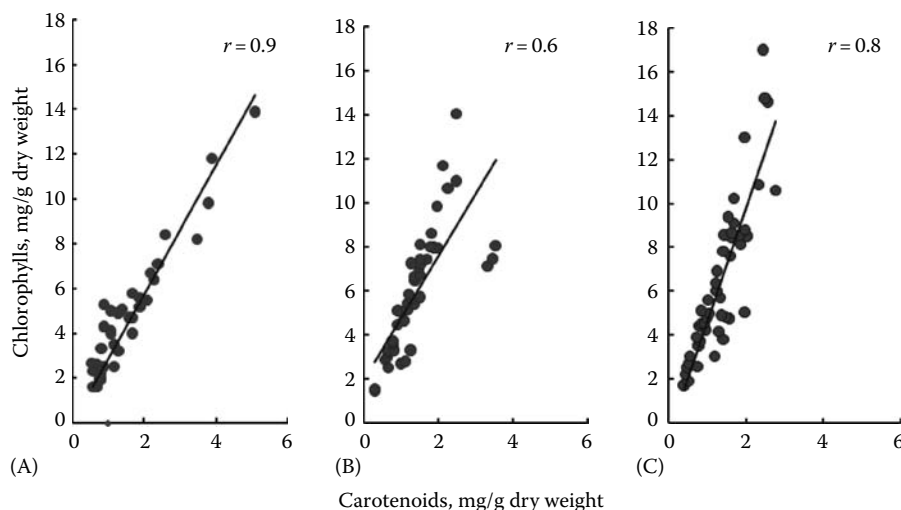


FIGURE 15.1 Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Subpolar Ural Mountains plants. For species names view according to the numbers in Table 15.2.

were characterized by a higher green pigment content. Conifer (*Larix sibirica*), dwarf shrubs (*Empetrum hermaphroditum*, *Phyllodoce caerulea*, *Ledum decumbens*, *Vaccinium uliginosum*), club mosses (*Lycopodium clavatum* and *L. annotinum*), and herbs (*Diapensia lapponica*) had a very low Chl content. In the leaves of more than 60% of the examined species, the content of green pigments consisted of 3–6 mg/g DW. These concentrations can be considered relatively low.

The Chl *a/b* ratio varied from 2 to 3.5, but it was higher (3.8–4.0) in *Bartsia alpina*, *Salix dasyclados*, *Astragalus frigidus*, and *Hedysarum arcticum*. In most of the species, Chl belonging to the light-harvesting complex (LHC-Chl) consisted of 55%–65% of the total. In *B. alpina*, *A. frigidus*, *H. arcticum*, and woody *S. dasyclados* leaves, the LHC-Chl values were the lowest (43%–45%).





**FIGURE 15.2** The relationship between chlorophylls and carotenoids in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), and the Middle Vychegda (C) plants.

The leaves of *Arctous alpine* and *Pyrola rotundifolia* were characterized by the highest LHC-Chl level (70%–80%).

There are positive correlations found between the yellow and the green pigment concentrations (Figure 15.2A). The majority of the examined species had the Car content of 0.9–1.7 mg/g DW. *Diapensia lapponica*, *Arctous alpine*, *Phyllodoce caerulea*, *Achillea nigrescens*, *Lycopodium clavatum*, *L. annotinum*, and *Vaccinium uliginosum* were distinguished by a low accumulation of yellow pigments (0.6–0.8 mg/g DW). Some of the legumes (*Hedysarum arcticum*, *Astragalus frigidus*) and *Pedicularis verticillata* had a high Car content, 4.5 mg/g DW. The Chl/Car ratios were 2.5–3.0 in most of the species, but these values were higher (4–5) in *Carex aquatilis*, *Calamagrostis purpurea*, and *Arctous alpine*.

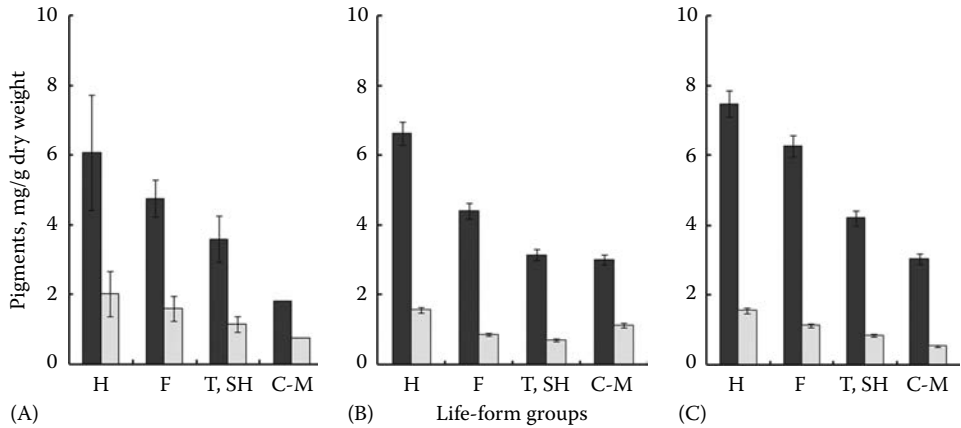
Plants with different Chl and Car accumulation levels were revealed among the groups that differed by life forms (herbs, ferns, trees and dwarf shrubs, and mosses). In the herbaceous plants group, there were species with an extremely low (*Achillea nigrescens*) and a high (*Astragalus frigidus*) content of photosynthetic pigments. However, overall perennial herbaceous plants, especially leguminous, contained more Chl and Car than trees and dwarf shrubs (Figure 15.3A). Among shrubs, *Salix* species, *Betula nana* and *Pentaphylloides fruticosa*, had a comparatively high Chl content (2–5 mg/g DW). Ferns had the same Chl level. Mosses were characterized by the lowest pigment accumulation.

A comparison of plants from different latitudinal groups (Figure 15.4A) showed that arctic, arctic-alpine, and hypo-arctic species contained similar amounts of photosynthetic pigments as that of the boreal species. In the arctic and arctic-alpine species, the ratio of green and yellow pigments was lower (2.9) as compared to boreal species (3.2). This indicates a relatively high Car in the pool of photosynthetic pigments of arctic and arctic-alpine species on the Subpolar Ural Mountains.

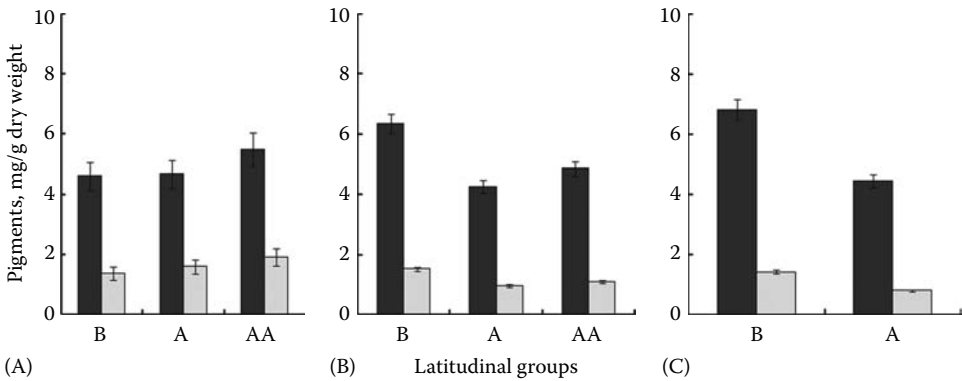
In general, the study carried out on the Subpolar Ural Mountains showed that the contents of the photosynthetic pigments depend more on the plant species and its life form, whereas the Chl/Car ratio depends on the geographical group. It should be noted that the pigment complex was characterized by a relatively low Chl content.

### 15.2.2 PIGMENT COMPLEX OF SOUTH TYMAN PLANTS

Among the 47 species of South Tyman plants examined, 74% belong to the boreal latitudinal group. The other species are included in the arctic, the arctic-alpine, and the hypo-arctic groups. These plant species were mostly found in rocky floristic complexes.



**FIGURE 15.3** Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), the Middle Vychegda (C). Life-form groups: H—herbs, T—trees, SH—shrubs, F—ferns, and C-M—club-mosses.

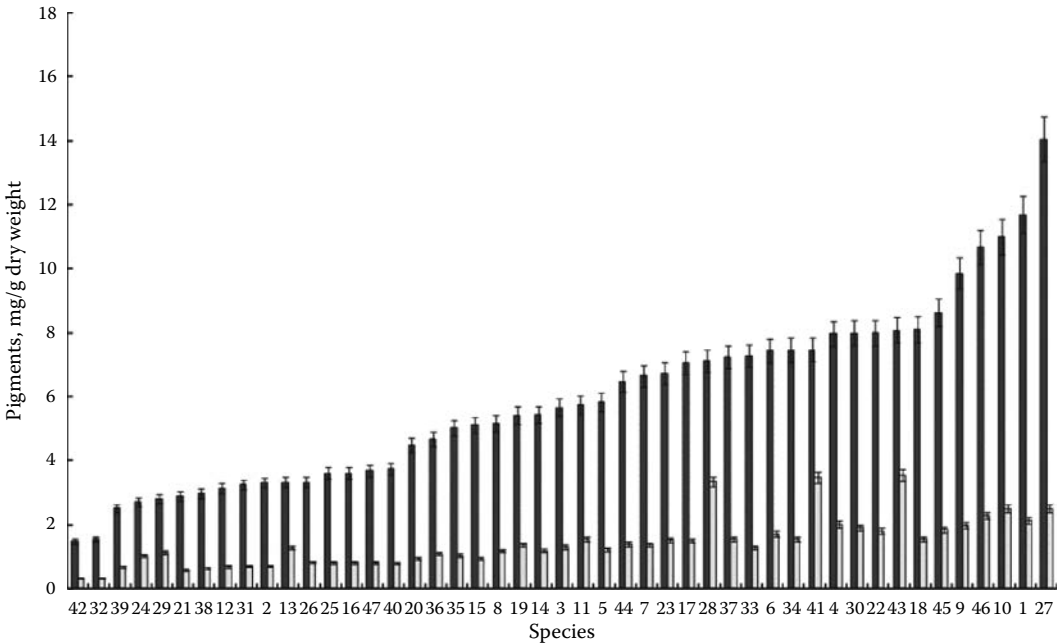


**FIGURE 15.4** Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the Subpolar Ural Mountains (A), the South Tyman (B), and the Middle Vychegda (C). Latitudinal groups: B—boreal, A—hypo-arctic, AA—arctic and alpine.

The South Tyman plants differed appreciably by the green pigment content. The Chl concentrations of these plant species varied from 1.5 to 14 mg/g DW (Figure 15.5). Conifer (*Pinus sibirica*), shrubs (*Vaccinium uliginosum*) and some herbs (*Pedicularis verticillata*, *Dendranthema zawadskii*) had comparatively low pigment content. Several species, including Trilliaceae (*Paris quadrifolia*), Orchidaceae (*Cypripedium calceolus*), Asteraceae (*Crepis sibirica*, *Petasites radiatus*), Rosaceae (*Geum rivale*), and Fabaceae (*Vicia cracca* and *V. sylvatica*, *Lathyrus pratensis*) were characterized by high Chl and Car contents.

The Chl and Car ratios were 2.4–3.1 in most of these species. These values were higher (3.6–3.9) in *Gymnadenia conopsea*, *Pedicularis verticillata*, and *Thymus taljievii* plants. The majority of species had LHC-Chl levels equal to 50%–65% of total Chl. Among the examined species, only in the leaves of *Pinguicula vulgaris*, LHC-Chl was 75%. The high percentage of Chl in LHC can increase the absorption of light energy and compensate a relatively low level of the green pigment in *Pinguicula* leaves. It should be noted that this plant was characterized by various types of nutrient deficiencies. It is an insectivorous plant.

As for the Subpolar Ural Mountains plants, a strong positive relationship between the content of the green and the yellow pigments with that of the South Tyman plants was revealed (Figure 15.2B).



**FIGURE 15.5** Chlorophyll (dark symbols) and carotenoid (light symbols) contents in the leaves of the South Tyman plants. For species names see according to the numbers in Table 15.2.

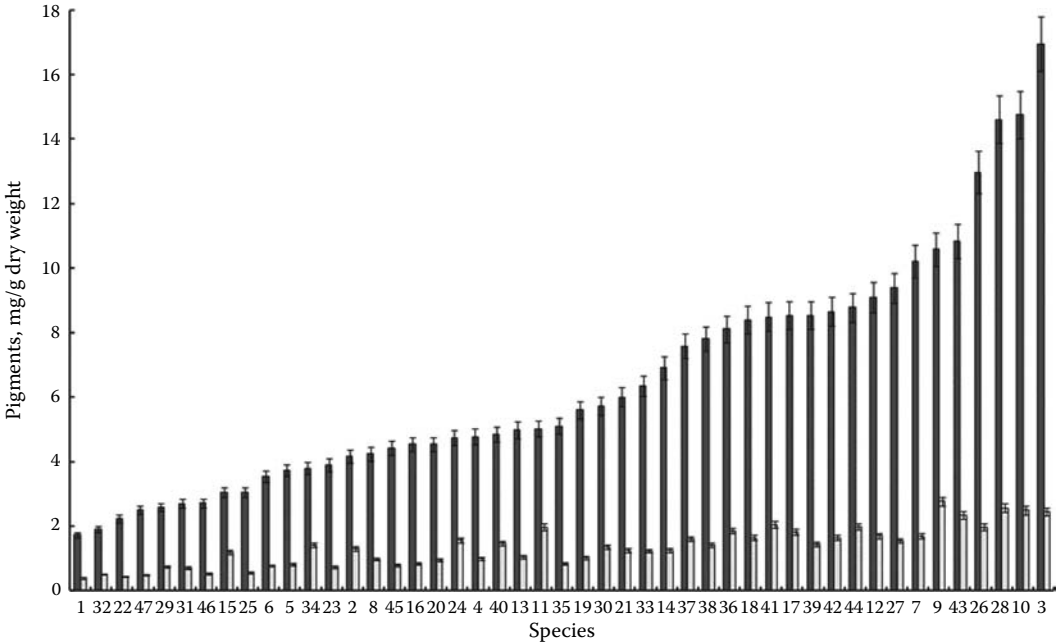
Although the Car concentration varied strongly, from 0.3 to 2.5 mg/g DW, the ratio Chl/Car was rather similar for most of the plants, i.e., equal to 4–5.

The herbaceous plants were characterized by higher Chl and Car contents than ferns, trees, and shrubs. The mosses had the lowest concentration of photosynthetic pigments (Figure 15.3B). The comparison of the plants from different latitudinal groups showed that arctic and hypo-arctic species accumulated lower amounts of pigment than the boreal plants (Figure 15.4B). Thus, arctic-alpine species were characterized by a low value of Chl/Car ratio (3.7) as compared to boreal plants (4.1).

### 15.2.3 PIGMENT COMPLEX OF MEADOW AND FOREST PLANTS IN THE MIDDLE VYCHEGDA BASIN

Forty-seven species inhabiting the meadows and forests were studied. Our data showed (Figure 15.6A) that the leaf Chl content was relatively high (more than 4–5 mg/g DW) in a majority of these plants. In *Paris quadrifolia*, *Calla palustris*, and *Aconitum septentrionale*, the Chl content was about 14 mg/g DW. The LHC-Chl level was varied significantly, from 44% to 74%. More LHC-Chl was accumulated in the forest herbs compared to the rest of the other species examined in this area. Although the species were distinguished by the Chl and Car concentrations, there was a positive correlation between the green and the yellow pigment accumulations. The content of the yellow pigments was four- to eightfold less than that of the green pigments. *Lycopodium clavatum*, *Vaccinium vitis-ideae*, *V. myrtillus*, *Abies sibirica*, and *Pinus sibirica* contained a comparatively small amount of Car (0.4–0.5 mg/g DW). But, *Thalictrum simplex*, *Alisma plantago-aquatica*, and *Aconitum septentrionale* accumulated five times more yellow pigments than green pigments.

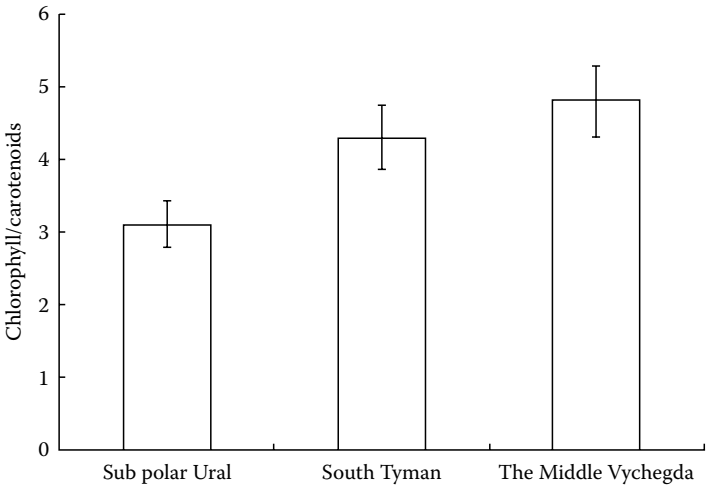
In the Middle Vycheгда flora, like the other regions, it was observed that the herbs were characterized by the highest accumulation of photosynthetic pigments (Figure 15.3C). The Chl and Car contents were 1.5–2-fold lower in trees and mosses. The comparison of the plants from different



**FIGURE 15.6** Chlorophylls (dark symbols) and carotenoids (light symbols) content in the leaves of the Middle Vychegda basin plants. For species names see according to the numbers in Table 15.2.

latitudinal groups showed that hypo-arctic species accumulated 1.3 times lower photosynthetic pigments than the boreal species (Figure 15.4C).

So, our data have shown that most of the examined plants from the Subpolar Ural Mountains and the South Tyman regions were characterized by a relatively higher content of the yellow pigments (Figure 15.7). The herbs accumulated 2–3 times more pigments than trees and mosses. Photosynthetic pigment pools in the leaves of the plants inhabited in the Middle Vychegda basin were larger as compared to plants on the Subpolar Ural Mountains.

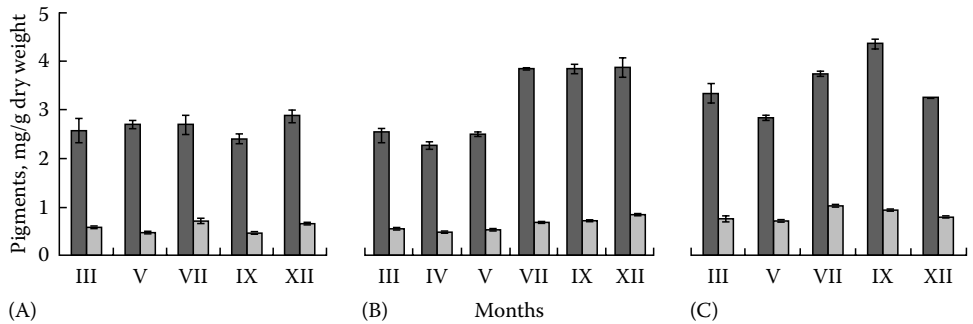


**FIGURE 15.7** The ratio of chlorophylls to carotenoids in the leaves of the plants from different regions.

15.3 SEASONAL CHANGES IN LEAF PIGMENT CONTENTS AND XANTHOPHYLLS CYCLE ACTIVITY IN EVERGREEN CONIFEROUS SPECIES

The evergreen conifers are the main woody species in the taiga zone and they occupy about 80% of the woodland of the Komi Republic (Virgin spruce forests ... 2006). The retention and maintenance of the pigment complex's functional activity in the annual cycle are important for the evergreen species of the boreal zone, which adapt to the long-term cold period. Low-temperature conditions during winter can inhibit CO<sub>2</sub>-exchange and carbon photosynthetic metabolism, but low temperatures do not affect the ability of chlorophyll to absorb light. Thus, winter conditions can cause a severe imbalance between light absorption and its utilization via photosynthesis. In such conditions, plants use photoprotective mechanisms to deal with the excess light absorbed by chlorophyll. The key mechanism includes the xanthophyll cycle-dependent thermal energy dissipation of excess light within the light-harvesting complexes. It is shown that the de-epoxidation of the xanthophyll cycle pigments was significantly higher in evergreen plants during winter (Garcia-Plazaola et al. 2003, Verhoeven et al. 2005).

We studied seasonal changes in the photosynthetic pigment content in three conifers: *Abies sibirica*, *Picea obovata*, and *Juniperus communis*. The concentrations of Chl and Car were relatively low, 2.4–4.3 × 0.45–1.0 mg/g DW, respectively (Figure 15.8). In winter, the reduction of the photosynthetic pigment pool was maximal in *Juniperus communis* needles. In *Picea obovata* and *Abies sibirica*, the pool of green pigments is more stable during the year. Essential seasonal differences in the chlorophyll *a/b*, the Chl, and the Car ratio were not found (Table 15.3). In spring and summer, all species had a tendency to a higher LHC-Chl level.



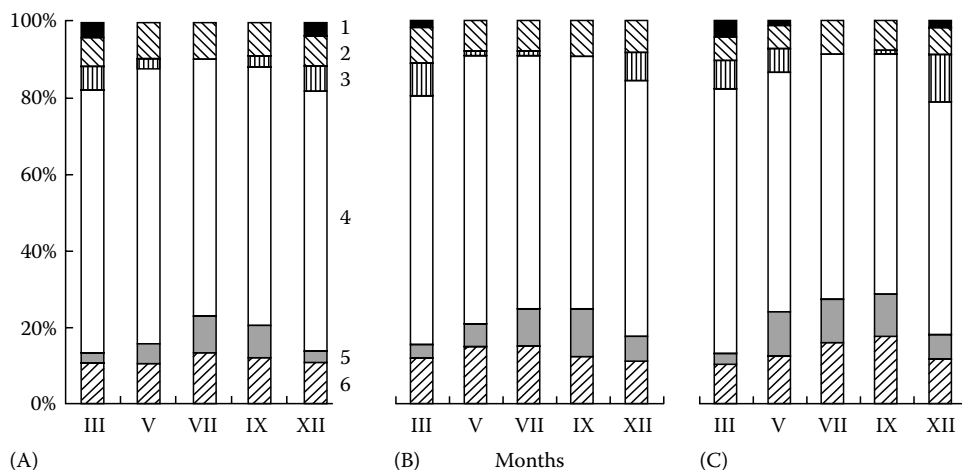
**FIGURE 15.8** Seasonal changes in the chlorophyll (dark symbols) and carotenoid (light symbols) contents in *Picea abies* (A), *Abies sibirica* (B), and *Juniperus communis* (C) needles. (From Yatsco, Y.N. et al., *Botan. Z.*, 94(12), 1812, 2009.)

**TABLE 15.3**  
**Chlorophyll and Carotenoid Composition of Evergreen Conifers Needles Sampled in Spring (III—March), Summer (VII—July), and Winter (XII—December), *n* = 5**

| Species                   | Chlorophyll <i>a/b</i> |           |           | LHC-Chl, % |     |     | Chlorophyll/Carotenoids |           |           |
|---------------------------|------------------------|-----------|-----------|------------|-----|-----|-------------------------|-----------|-----------|
|                           | III                    | VII       | XII       | III        | VII | XII | III                     | VII       | XII       |
| <i>Abies sibirica</i>     | 2.4 ± 0.1              | 2.5 ± 0.1 | 2.7 ± 0.1 | 65         | 64  | 59  | 4.6 ± 0.1               | 5.7 ± 0.1 | 4.6 ± 0.1 |
| <i>Picea abies</i>        | 2.4 ± 0.1              | 2.5 ± 0.3 | 2.9 ± 0.1 | 66         | 63  | 57  | 4.5 ± 0.3               | 4.5 ± 0.4 | 4.4 ± 0.1 |
| <i>Juniperus communis</i> | 2.8 ± 0.1              | 2.8 ± 0.1 | 2.9 ± 0.1 | 60         | 57  | 56  | 4.5 ± 0.1               | 4.2 ± 0.1 | 4.1 ± 0.1 |

Source: Yatsco, Y.N. et al., *Botan. Z.*, 94(12), 1812, 2009.

Note: LHC-Chl—Chlorophyll belonging to light-harvesting complex, *n* = 5.



**FIGURE 15.9** Seasonal changes in the carotenoid composition (as a percent of total carotenoid content) in *Picea obovata* (A), *Abies sibirica* (B), and *Juniperus communis* (C) needles. 1—zeaxanthin, 2—neoxanthin, 3—antheraxanthin, 4—lutein, 5—violaxanthin, and 6— $\beta$ -carotene.

The carotenoid composition for all species was presented mainly by xanthophylls (90%) during the whole year (Figure 15.9). The portion of  $\beta$ -carotene consisted of 10%–20% of Car content. Among the xanthophylls, the level of lutein was the highest and reached 70%. Neoxanthin (7%–10%) and violaxanthin (3%–15%) were presented constantly in relatively small amounts. In the studied plants, violaxanthin was the predominant component of the xanthophylls cycle, whereas two other components of the xanthophyll cycle (zeaxanthin and antheraxanthin), were noted mainly during winter and spring, and none identified in summer.

The analyses of the seasonal changes of the individual carotenoids showed an increase of  $\beta$ -carotene and violaxanthin levels in summer, whereas antheraxanthin and zeaxanthin were practically absent at this time. A low amount of antheraxanthin was found in autumn. Antheraxanthin levels in the carotenoid pools of *Picea obovata* and *Abies sibirica* needles were 7%–8% in winter and at the beginning of spring. There was 15% (of total carotenoid content) of antheraxanthin in *Juniperus communis* in winter. The level of antheraxanthin (up to 5%) was preserved in *Juniperus* needles until May. Zeaxanthin appeared in the beginning of winter, and its level increased in spring. Zeaxanthin disappeared fully in summer. The same seasonal pattern of zeaxanthin was revealed for *Picea obovata* and *Abies sibirica*.

The de-epoxidation state (DEPS) was characterized by the ratio between de-epoxidized and the epoxidized forms of the xanthophyll cycle components. According to Schindler and Lichtenthaler (1996), DEPS can be estimated from the equation,  $DEPS = (zeaxanthin + 0.5 \text{ antheraxanthin}) / (zeaxanthin + antheraxanthin + violaxanthin)$ . Evaluation of DEPS showed that the de-epoxidation of the xanthophyll cycle pigments was high in all the examined species in winter and the early spring (Table 15.4). The DEPS values decreased 2–3 times in May. The de-epoxidation of the xanthophyll cycle pigments was almost completely suppressed in summer (July). The repair of de-epoxidation reaction (by 30%) was observed in *Picea obovata* needles in autumn (September), in the other two species—in December. It should be noted that for this period, *Picea obovata* was already characterized by maximum DEPS values.

We noted already, that *Picea obovata* needles were distinguished by the greatest stability of the pigment pool during the year among the studied species. In *Abies sibirica* and *Juniperus communis* needles, a portion of the pigments in winter and spring had been destroyed. Several investigators have reported the loss of chlorophyll in the needles of the conifers, in winter (Ottander et al. 1995, Enslinger et al. 2004, Martz et al. 2007). Information about pigment complex preservation was

**TABLE 15.4**  
**Seasonal Changes in Deep-Oxidation State of**  
**Xanthophyll Cycle Pigments in Evergreen Conifer**  
**Needles, %, *n* = 5**

| Months    | <i>Picea obovata</i> | <i>Abies sibirica</i> | <i>Juniperus communis</i> |
|-----------|----------------------|-----------------------|---------------------------|
| March     | 54                   | 46                    | 60                        |
| May       | 12                   | 23                    | 21                        |
| July      | 0                    | 2                     | 0                         |
| September | 13                   | 0                     | 3                         |
| December  | 53                   | 30                    | 38                        |

given by Lukyanova et al. (1986). It seems that the different opinions about the character of the seasonal changes in pigment composition in conifers depend on the species traits and the climatic conditions of the habitats. Winter and spring loss of pigment was repaired in summer and autumn. The structural and functional changes of mesophyll at the initial stage of the hardening in autumn are important for the preservation of the photosynthetic apparatus. In the needles of *Picea obovata*, the disappearance of the starch grains, the change of form, size and localization of the chloroplasts are shown (Ladanova and Tuzhilkina 1992). The absorption of light energy was reduced due to the relocation of chloroplasts to the central part of the cell and grouping around the nucleus. This fact can be regarded as the adaptive reaction that is directed to the reduction of light absorption in winter and spring when the photosynthetic consumption of excitation energy is blocked.

**15.4 SUMMARY AND CONCLUSIONS**

The contents and the ratio of photosynthetic pigments in plant leaves, growing in the Subpolar Ural Mountains, the South Tyman, and the Middle Vychegda basin, were investigated. We found that the level of the carotenoid accumulation in the plant leaves from all the studied regions correlated closely with the chlorophyll content. In comparison of our data with the results reported by Maslova and Popova (1993) for other regions, it was found that the Subpolar Ural Mountains plants are closer to the alpine plants of the East Pamirs, the South Tyman plants—to the arctic plants of Taimir, and the middle Vychegda plants—to the species of the temperate zone on the Chl/Car index.

Carotenoids exhibit both light-harvesting and photoprotective functions (Demmig-Adams 1990, Rmiki et al. 1999, Cuttriss and Pogson 2004). Therefore, the relatively high level of the yellow pigments in the leaves of the Northern plants, especially arctic species, can be regarded as the adaptive reaction that is directed to an increase in the stability of the pigment complex and to prevent its photodynamic destruction in the cold climate. The pigment complex of the species from the middle taiga subzone was characterized by a higher Chl/Car ratio and the lower yellow pigment contents were in contrast to the Subpolar Ural Mountains plants. This confirms the important role of the carotenoids in the stability of the pigment complex of the extremely north taiga subzone plants. It should be noted that carotenoids not only have a protective role, but also absorb light in the near ultraviolet (UV) as well as the visible region. Carotenoids are bound, together with Chl, to proteins and participate in light harvesting. As components of the antenna complex, they provide more effective use of solar radiation during the short north summer.

Pigments, especially carotenoids, perform an essential photoprotective role in evergreen conifers' photosynthetic apparatus. Preservation and the long-term functioning of the photosynthetic apparatus allow conifers to occupy the temperate and subarctic Northern hemisphere, where freezing temperatures occur regularly and cold acclimation processes are essential to enable plants to withstand periods of very low temperatures (Levitt 1980). Conifers retain their needles for several

years and so their photosynthetic apparatus must survive severe freezing periods, often combined with high light, especially in early spring. The interaction of light and low temperature can result in the photo-inhibition of photosynthesis in nature (Martin et al. 1978, Ottander and Oquist 1991). Our data showed that the pool of carotenoids in conifers during the vegetative period consists of  $\beta$ -carotene, lutein, and violaxanthin. In pigment apparatus of the conifers, the high level of lutein remained fairly constant during the year. It is possible that the lutein content is related to the structural role of this xanthophyll, which is the integral component of the peripheral unit of LHC II, and on its possible antioxidant function (Kuhlbrandt et al. 1994). Accumulation of antheraxanthin and zeaxanthin in the carotenoid spectrum was observed in winter and at the beginning of spring under freezing stress combined simultaneously with high light. According to (Demmig-Adams 1990, Eskling et al. 1997) zeaxanthin is the exclusive xanthophyll that is accumulated under excess light by deep oxidation of the existing violaxanthin in the xanthophyll cycle. It is widely thought to play a photoprotective role by the dissipation of excessive light energy as heat (Niyogi 1999). Previous research has shown that zeaxanthin can quench the  $^1\text{Chl}^*$  state directly through the process of non-photochemical quenching (NPQ) (Muller et al. 2001). Antheraxanthin, another xanthophyll, has a similar function of photoprotection with zeaxanthin, and it can replace zeaxanthin in the absence of zeaxanthin (Goss et al. 1998). So, our data show that the primary photoprotective mechanism of the three evergreen conifers growing in the cold climate is the increase in the degree of the de-epoxidation state of the xanthophyll cycle and the accumulation of zeaxanthin. It provides a harmless dissipation of excess excitation energy in the photochemical system. Lutein, another xanthophyll, is important for photoprotection in conifers too.

As a whole, information on pigment apparatus of different botanical and ecological groups of Northern plants was completed and summarized. The significant differentiation of species on the green and the yellow pigment contents was found. An increase of the relative content of carotenoids in row boreal—hypo-arctic—arcto-alpine species was found. New data on the light-dependent changes of xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) on the Northern conifers are found. The role of carotenoids in the tolerance of photosynthetic apparatus and protection against photo-oxidative damage in leaves of plants from the Northern regions is shown. It was concluded that the relatively high content of carotenoids plays a major role in the stability of the photosynthetic apparatus in cold climatic conditions. The results obtained extend our knowledge about mechanisms of adaptation of pigment apparatus and photosynthetic function realization in plants under cold climate environments.

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# 16 Modifications of the Carotenoid Metabolism in Plastids: A Response to Stress Conditions

*Pascale Moulin, Yves Lemoine, and Benoît Schoefs*

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## 16.1 INTRODUCTION

Since the emergence of life on Earth, the food chains depend on the use of solar energy by photosynthetic organisms. One characteristic feature of these organisms resides in their global lack of mobility, even though microalgae can move in the water column. Consequently, this type of organisms cannot run away from the alterations in their biotic and/or abiotic environments, including deficiencies or excesses of light or nutrients. To cope with these alterations, photosynthetic cells activate different types of defense mechanisms, including reorientations of the plastid metabolism. Because the rapidity and efficiency of the responses differ among species or even plant cultivars, this aptitude to adapt to unfavorable conditions constitutes a major factor for their survival (Külheim et al. 2002). For instance, sun-grown leaves exhibit a greater ability to activate photoprotective mechanisms, such as the xanthophyll cycle, than shade-grown plants when both types of plants are exposed to high light (Demmig-Adams and Adams 1994, 1995, 2006).

In this chapter, that is an update of the data presented in the second edition of the *Handbook of Plant and Crop Stress* (Rmiki et al. 1999), we have reviewed the data dealing with the changes in the carotenoid (Car) and apoCar (ApoCar) metabolisms in algae and plants as a response to stress.

## 16.2 PROPERTIES OF CAROTENOIDS IN PHOTOSYNTHETIC ORGANISMS

Cars are C40-polyisoprenic compounds characterized by a large number of conjugated double bonds ( $n > 7$ ). More than 600 Cars have been identified to date and several new ones are reported annually. Conjugated double bonds allow Cars to absorb light in the near-ultraviolet as well as in the visible region. In photosynthetic organisms, Cars are bound, together with chlorophylls (Chls), to proteins and participate in light harvesting and in energy transfer to the photosynthetic reaction centers. This last process resides on a very efficient singlet–singlet energy transfer from Cars to Chls and needs a precise arrangement of pigment molecules in the light-harvesting complexes (LHCs). Cars are also recognized to be essential for the survival of illuminated plants, since their numerous conjugated double bonds are able to quench the Chl triplet state and also to scavenge singlet oxygen and the other reactive oxygen species (ROS), which are abundantly produced during photoinhibition. This photoprotective function is generally achieved *via* triplet–triplet energy transfer. In addition to these well-established functions, Cars of higher plant plastids might play important structural roles in stabilizing the lipid phase of the thylakoid membranes through modification of the membrane fluidity (for a review, see Havaux 1998).

## 16.3 DIVERSITY OF STRESSES ON CAROTENOID BIOSYNTHESIS

Various factors (e.g., nutrient deficiency, excess light, drought, and chilling) are known to have consequences on the photosynthetic apparatus, and photoinhibition may be often observed under these environmental conditions. Actually, in their natural environment, plants are constantly confronted with reconciling an excessive energy supply with the demands of the photosynthetic carbon reduction cycle for the products of electron transport, ATP and NADPH (Osmond 1981). When light absorption by LHC exceeds both the capacity to use the photosynthetic NADPH and ATP for carbohydrate synthesis and the capacity of energy dissipation mechanisms, photosynthesis is progressively inhibited (i.e., photoinhibition phenomenon). This means that even low light levels may become excessive if combined with chilling and then may result in photoinhibition in crops or in algae. The consequences at the Car level of any stress may be multiple (for a review, see Young and Britton 1990).

To cope with the absorption of excessive light and its consequences, the photosynthetic organisms have evolved a series of short-term (10–30 min) and long-term photoprotective mechanisms (Horton and Ruban 1992). Among these mechanisms, the thermal dissipation of excess absorbed light energy at the photosystem (PS) II, the so-called nonphotochemical quenching (NPQ), is believed to play a key role in regulating the light harvesting and in the prevention of photooxidative damages.

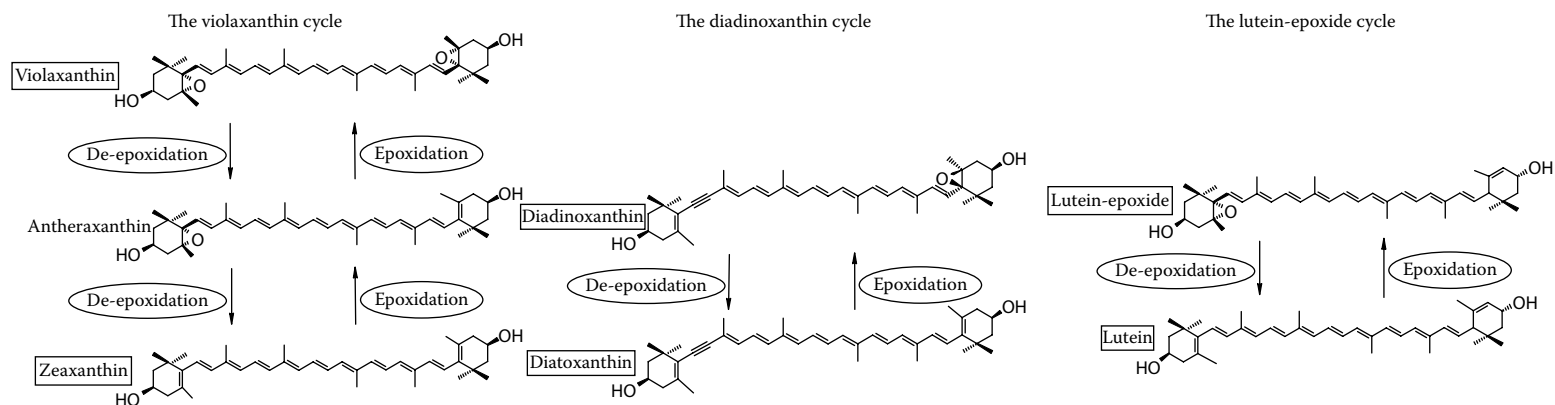
## 16.4 THE VIOLAXANTHIN CYCLE: A SHORT-TERM PHOTOPROTECTIVE MECHANISM

Basically, the photosynthetic apparatus constitutes an optimized biological device to store the energy of light into molecules. Because the photosynthetic apparatus manipulates oxygen and light, the interaction between singlet Chl molecules and triplet oxygen is strictly avoided by a variety of photoprotective Cars bound to the proteins of the photosynthetic apparatus (Frank and Cogdell 1996), because the interaction yields to the formation of singlet oxygen, which can damage proteins, pigments, and lipids in the photosynthetic apparatus (Niyogi 1999, Asada 2006). When the capacity to harvest the light energy is higher than the capacity to use it, the actual set of photoprotective Cars is no longer able to efficiently dissipate the absorbed energy and the excess of absorbed light should be dissipated through other pathways. Early research identified a dissipation process induced by the lowering of the lumen pH, a condition triggered by high-light conditions (for reviews, see Krause and Weis 1991, Demmig-Adams and Adams 1996, Rmiki et al. 1999). A connection was subsequently made between energy dissipation and the Cars of the xanthophyll cycle (Demmig et al. 1987, Demmig-Adams 1990). The role of zeaxanthin (Zea) as a general antioxidant has been confirmed using the *npq2* mutant that accumulates Zea (Niyogi et al. 1998) and shows a higher NPQ level than in the wild type (Niyogi et al. 1998, Havaux et al. 2000, Kalituho et al. 2007). Studies of *Arabidopsis* plants containing nearly three times the amount of xanthophyll cycle Cars present in the wild type have given further details on the antioxidant function of these pigment conversions involving their binding to proteins of the LHC family (Johnson et al. 2007).

With the development of a high-pH gradient across the thylakoids, two-steps mono de-epoxidation reactions of the Violaxanthin (Vio) into Zea with antheraxanthin (Ant) as an intermediate are catalyzed by the Vio de-epoxidase (VDE) (EC 1.10.99.3) (Figure 16.1). The Vio molecules are regenerated through two light-independent epoxidation steps that are catalyzed by the Zea epoxidase (ZEP) (EC 1.14.13.90). The transformation of Vio to Zea proceeds in about 10–30 min (Sierfermann-Harms 1977) whereas the conversion of Zea to Vio is 5–10 times slower (Härtel et al. 1996, Marin et al. 1996). Both VDE and ZEP have a similar basic tertiary structure, maybe because they share a common Ant substrate and belong to the lipocalin family of proteins (Bugos et al. 1998; Hieber et al. 2000, 2002, Wang et al. 2008; for a review, see Jahns et al. 2009). Lipocalins are characterized by a similar tertiary structure and similar functions (Grzyb et al. 2006). These enzymes are supposed to contain eight antiparallel  $\beta$ -sheets and three highly conserved short consensus repeat motifs (Flower et al. 2000). The motif I is composed of the first of the eight  $\beta$ -sheets and a short fragment of the preceding  $\alpha$ -helix. Motif II is composed of the loop between  $\beta$ -sheets number 6 and 7 and parts of the end of  $\beta$ -sheet number 6 and the beginning of  $\beta$ -sheets number 7. The motif III is composed of the end of  $\beta$ -sheet number 8 and part of the C-terminal  $\alpha$ -helix, including the loop between both fragments. VDE and ZEP are conserved in two and only one short consensus repeat motif, respectively (Akerstrom et al. 2000), and differ by the number of amino acid residues between the motif I and II (Bugos et al. 1998). The 3D structure of lipocalins is characterized by the deep conic hollow, formed by the  $\beta$ -sheets. The deep conic hollow is necessary for substrate binding (Newcomer et al. 1984, Holden et al. 1987).

### 16.4.1 VIOLAXANTHIN DE-EPOXIDASE

The first cDNA sequence of VDE was obtained from romaine lettuce (Bugos and Yamamoto, 1996). The VDE sequences homologous to VDE from plants were identified in the genome of diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Bacillariophyceae). No VDE sequence was found in the red algae genome library even if some red seaweeds are able to produce Ant (Rmiki et al. 1996) or Vio (Coesel et al. 2008). For instance, *Gracilaria gracilis* and *G. multipartita* are devoid of Vio, and a xanthophyll cycle restricted to the interconversions of Ant to Zea was observed (Rmiki et al. 1996; Garcia-Plazaola et al. 2007). A sequence constituting a novel gene,



**FIGURE 16.1** The different xanthophyll cycles. Under stress conditions, Vio, Ddx, or Lut-epoxide are de-epoxidized into Zea, Dtx, or Lut, respectively. After suppression of the stress conditions, the de-epoxidated xanthophylls are epoxidized.

named VDE-related or VDR, was identified in the genome of *Chlamydomonas reinhardtii* as sharing a low sequence similarity with *Arabidopsis thaliana* VDE. VDE-related homologs were found in higher plants and in two diatom genomes (Coesel et al. 2008).

VDE is a nucleus-encoded protein. The plastid import mechanism involves a bipartite transit peptide located at the N-terminal of the preprotein sequence. The first part of the transit peptide allows the targeting of the protein to the chloroplast stroma, whereas the second part drives the thylakoid-targeting. These results confirm the location of mature VDE protein (43 kDa) in the lumen of the thylakoids (Bugos et al. 1999; for a review, see Rmiki et al. 1999). Depending on the pH, the mature VDE, that is present in very small amounts, occurs either as a soluble protein (neutral pH) or as a tightly bound protein at the lumenal surface of the thylakoid when the lumen acidifies (Eskling et al. 1997). The mutant *npq1* is defective in VDE (Niyogi et al. 1998).

The comparison of the VDE sequences showed similarities at two domains. The first one is a cystein-rich N-terminal domain, probably organized in  $\alpha$ -helices, whereas the second one is a glutamic acid-rich C-terminal domain, composed of long  $\alpha$ -helices (Hieber et al. 2000). This last domain would serve for the pH-dependent binding. Actually, when it is partially protonated, the binding of VDE protein to the thylakoid membrane is increased (Hieber et al. 2002, Coesel et al. 2008). In VDE, the lipocalin hollow fits with the length of Vio molecule and would explain why there is strict specificity of the enzyme for 3-OH, 5-6-epoxy Cars in configuration 3*R*, 5*S*, and 6*R* (Yamamoto 1979, Grotz et al. 1999).

VDE requires ascorbate, under its acidic form, as a cosubstrate (Yamamoto 1979, Eskling et al. 1997, Jahns et al. 2009). The protonated ascorbate content of the lumen is an endogenous proton and electron donor and it has been shown to activate the VDE activity *in vivo* in *Arabidopsis* (Yamamoto 1979, Neubauer and Yamamoto 1994, Müller-Moulé et al. 2002). The optimum ascorbate concentration for VDE activity is pH-dependent, that is, at pH 4.5–5.5, the enzyme becomes saturated at 10–20 mM, whereas at pH 6, the saturation level is not reached before 100 mM (Bratt et al. 1995). Ascorbate, reduced in the stroma by the glutathione cycle, which consumes NADPH and glutathione, may cross the thylakoid membrane and regulate the activity of PSII (Foyer et al. 1989). The existence of a membrane transporter for negatively charged ascorbate in exchange for dehydroascorbate was postulated (Bratt et al. 1995, Eskling et al. 1997).

The VDE activity is reversibly inhibited by dithiothreitol through the reduction of the disulfide bonds in the enzyme if the inhibitor is added at pH 5.2–5.7 (Yamamoto and Kamite 1972, Bugos et al. 1999, Coesel et al. 2008).

#### 16.4.2 ZEAXANTHIN EPOXIDASE

The first ZEP cDNA, originally named as ABA2, was isolated from tobacco (*Nicotiana glauca*) using the insertional mutagenesis technique (Marin et al. 1996, Audran et al. 1998, Wang et al. 2008). Later, this cDNA was used to allow the isolation of other ZEP sequences from other dicotyledon plants like pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), *Arabidopsis*, and apricot (*Prunus armeniaca*) (Hieber et al. 2000), monocotyledon plants, green algae, diatoms, and *Tetrahymena thermophila*—a ciliate organism (Coesel et al. 2008). The mutant *npq2* of *A. thaliana* is devoid in ZEP (Niyogi et al. 1998).

ZEP preprotein contains a chloroplast transit peptide that is cleaved during the targeting into the chloroplast stroma (Audran et al. 1998). The comparison of ZEP mature protein sequences indicated that, in addition to the lipocalin typical domain, the sequences also share a flavoprotein monooxygenase consensus domain similar to the prokaryotic aromatic-substrate monooxygenases (Hieber et al. 2000). The sequence has a phosphopeptide-binding domain, which could be involved in protein–protein interactions (Coesel et al. 2008, Wang et al. 2008). The ZEP enzyme, that is active at the stromal side of thylakoids, with an optimum pH around 7.5, requires FAD, O<sub>2</sub>, NADPH, and “ferredoxin-like” reductives as cosubstrates for the epoxidation reaction (Hager 1975, Siefermann and Yamamoto 1975a, Siefermann and Yamamoto 1975b, Siefermann and Yamamoto 1975,

Wang et al. 2008). So far, no ZEP protein could be purified from organisms. Actually, the regulation of ZEP activity is not well understood but it has been shown that downregulation may occur under extreme photooxidative stress conditions in the short- (Jahns 1995, Reinhold et al. 2008) or long term (Adams et al. 2002).

Even if the ZEP tomato gene was expressed preferentially in chlorophyllous tissue, the ZEP transcripts were also detected in flowers, roots, and fruits (Wang et al. 2008). In the nonchlorophyllous tissues of *Arabidopsis*, Zea epoxidation is involved in the abscissic acid biosynthesis (Audran et al. 2001). Diatoms that do not produce this phytohormone also use ZEP to synthesize fucoxanthin, their major light-harvesting Car, is realized at the expense of Vio (Lohr and Wilhelm 2001).

### 16.4.3 SECOND XANTHOPHYLL CYCLE IN HIGHER PLANTS: THE LUTEIN-5,6-EPOXIDE CYCLE

In maturing tomato fruit (*Lycopersicum esculentum* Mill. cv Moneymaker), besides a typical Vio cycle, a second cycle of lesser amplitude was observed between Lutein (Lut) and Lut monoepoxide (Rabinowitch et al. 1975) (Figure 16.1). More recently, operation of this new type of xanthophyll cycle was observed in two parasitic angiosperms, that are, *Cuscuta reflexa* (Bungard et al. 1999) and *Amyema miquelii* (Matsubara et al. 2001) but not in the roots of other parasitic organisms such as *Arceuthobium* sp. and *Phoradendron* sp. (Ladygin 2008). This cycle was later found in maturing green fruits of tomato and in leaves of eight *Quercus* species (Garcia-Plazaola et al. 2002a). A de-epoxidase is involved in this cycle allowing, under a strong light, the conversion of Lut-5,6-epoxide into Lut, whereas under low light, the reverse reaction goes back but at a very slow rate. One important question that remains is to determine which enzymes are involved in the Lut-Lut-epoxide cycle. In the one hand, no enzyme implicated in the Lut-5,6-epoxide cycle was clearly identified (Giuliano et al. 2008) but in the other hand, VDE could catalyze, in addition to the Vio-de-epoxidation, the conversion of Lut-5,6-epoxide into Lut *in vitro* (Grotz et al. 1999, Goss 2003, Ladygin 2008). In addition, the  $\beta$ -ring of Lut may be a substrate for ZEP (Hieber et al. 2000). Thus, the Lut-epoxide cycle is probably driven by the same set of enzymes that the Vio cycle.

The species in which the Lut-epoxide cycle has been studied range into three groups according to their Lut-epoxide cycle pool of pigments (for more details, see Garcia-Plazaola et al. 2007): (i) species such as *Quercus subpyrenaica* (Abadia et al. 1996), *Q. ilex* (Llorens et al. 2002), and *Viscum album* (Matsubara et al. 2003) have very low amounts (1–10 mmol mol<sup>-1</sup> Chl) and Lut-epoxide/VioAntZea ratio < 0.1; (ii) species such as *Q. robur* (Garcia-Plazaola et al. 2002a), the mistletoe *A. miquelii* Thiegh (Matsubara et al. 2003), and *Acacia melanoxylon* (Watson et al. 2004) contain high amounts of Lut-epoxide and the Lut-epoxide/VioAntZea ratio ranges between 0.1 and 1; and (iii) leaves from shade plants such as the tropical tree *Inga sapindoides* (Matsubara et al. 2005), *Laurus nobilis*, and *Umbellaria californica* (Estaban et al. 2007) have a high pool of Lut-epoxide, up to 78 mmol mol<sup>-1</sup> Chl with a Lut-epoxide/VioAntZea > 1. The frequent Lut-epoxide accumulation in shade leaves relies on a slow epoxidation of Lut by ZEP and on the absence of VDE activity in such conditions. The extremely slow conversion of Lut to Lut-epoxide has been suggested to result from a low affinity of ZEP for Lut (Pogson and Rissler 2000) or it could be due to the low availability of this substrate, which is mainly restricted to the intraprotein Lut sites of Lhcb (Morosinotto et al. 2002, Dekker and Boekema 2005). In their review on the occurrence and possible functions of the Lut-epoxide cycle, Garcia-Plazaola et al. (2007) proposed a model describing the relationships between the Lut-epoxide and Vio cycles. In deep shade, both Vio and Lut epoxide may occupy the Vio1 site while the Lut2 site may be occupied by Lut-epoxide instead of Lut, giving rise to very efficient light-harvesting PSII antenna. After exposure to strong light, the displacement of Lut-epoxide by Lut in Vio1, Lut2, and most Lut1 sites gives efficient energy dissipation centers. A greater pool of Lut derived from Lut-epoxide de-epoxidation as compared with Ant and Zea derived from Vio offers the possibility of a rapid and strong engagement of NPQ. Photoprotection may then be stabilized within hours of exposure to strong light by simultaneous engagement of both cycles, the slowly reversible conversion of Lut being proposed to “lock-in” a



primary mechanism of photoprotection (Matsubara et al. 2005). Thus, the Lut-epoxide cycle could serve as an additional, more slowly relaxing mechanism, to accelerate and sustain the development of NPQ (Garcia-Plazaola et al. 2003).

#### 16.4.4 SPECIFIC XANTHOPHYLL CYCLE INVOLVED IN DIATOMS, XANTHOPHYTES, DINOPHYTES, AND HAPTOPHYTES: THE DIADINOXANTHIN CYCLE

The diadinoxanthin (Ddx) cycle involves the Ddx de-epoxidase (DDE), which de-epoxidises the epoxy-xanthophyll Ddx, whereas the Dtx epoxidase (DEP) catalyzes the back reaction (Wilhelm et al. 2006, Garcia-Plazaola et al. 2007) (Figure 16.1). Several publications have reported on the detection and the regulation of DDE and DEP activities in diatoms (Jakob et al. 2001, Goss et al. 2006, Kroth 2007). Homologous sequences to *DDE* and *DEP* genes were found in the genome database of two diatoms, *T. pseudonana* and *P. tricornutum*, and were named VDE-like because they are distantly related to the plant VDE. VDE-like enzyme could be located differently than the VDE protein because the C-terminal region, which could be important for binding the protein to the thylakoid membrane, is uncharged. Coesel et al. (2008) have proposed that VDE-like enzyme could be implicated in the Ddx cycle of these diatoms. In the same way, the *ZEP* gene product is implicated in the reverse reaction. Compared with the enzymes involved in the Vio cycle, much less is known about the Ddx cycle enzymes from algae. The DDE, homologous of VDE, exhibits a different pH-dependance for its activation, which is shifted by at least 0.7 pH unit toward higher pH values (Jakob et al. 2001). The affinity of DDE for the cosubstrate ascorbate is three to four times higher than in the case of VDE (Grouneva et al. 2006). DEP, which catalyzes the back conversion of Dtx to Ddx is almost inhibited under high-light conditions (Mewes and Richter 2002) and can be also inhibited by cadmium (Bertrand et al. 2001, for reviews see Bertrand and Poirier 2005, Poirier et al. 2008; see also the chapter by Solymosi and Bertrand).

Lohr and Wilhelm (1999) have shown that besides the Ddx cycle, some diatoms may also display a Vio cycle, even if the pool size of pigments concerned with the Vio cycle is rather low.

Another xanthophyll cycle has been proposed to operate in green algae, involving L-siphonaxanthin, a xanthophyll present in the siphonaceous genera, in *Ulva olivescens* (Levavasseur 1981) and in a few Prasinophyceae (Latasa et al. 2004). In *Codium fragile* and *Cladophora opaca*, Vershinin and Kamnev (1996) observed a reversible light-induced de-esterification of siphonaxanthin dodecenoate ester (wrongly identified as siphonoin B (Britton et al. 2004)), with siphonaxanthin accumulation in high light and siphonaxanthin dodecenoate ester in low light. More recently, Raniello et al. (2006) described another xanthophyll cycle operating in addition to the Vio cycle in the shallowest populations of the invasive alga *Caulerpa racemosa* var. *cylindracea*. Interconversions between Lut and siphonaxanthin were shown to occur along the day with Lut and siphonaxanthin highest content under high- and low irradiances, respectively. No variations of the siphonaxanthin dodecenoate ester content could be observed by these authors. Thus, besides the conventional Vio and Ddx cycles, which have been exhaustively studied along the last 40 years, several other xanthophyll cycles have been described more recently, which often operate in parallel with the Vio cycle and participate to photoprotection.

#### 16.4.5 XANTHOPHYLL CYCLES AND EVOLUTION OF PLANTS AND ALGAE

As no homolog of VDE protein have been found in prokaryotes, it could be possible that this protein represents an ancient eukaryotic innovation (Coesel et al. 2008). Actually, cyanobacteria cells seem to be totally devoid of any xanthophyll cycle (Lemoine et al. 1993), even if many taxons, such as *Spirulina*, have a high Vio content. However, the lipocalin protein membership of VDE and ZEP suggests that they may have evolved from lipocalin genes of cyanobacterial ancestors that developed an endosymbiotic relationship with a eukaryotic host cell to form the chloroplast of algae and

higher plants (Durnford et al. 1999, Palmer 2003, Charron et al. 2005). The presence of Vio and its precursors Ant and Zea was frequently observed among Rhodophyte species (Schubert et al. 2006) and operation of a total Vio cycle was proved in *G. birdiae* (Ursi et al. 2003). Even if the only Rhodophyte species in which the Vio cycle was reported belong to the Gracilariales (Rmiki et al. 1996, Ursi et al. 2003), the Vio cycle pigments were also detected in other orders such as the Ceramiales and the Corallinales (Schubert et al. 2006). These observations are important on an evolutive point of view because they suggest that the epoxy-Cars involved in the Vio cycle were present early in the evolution of red algae, before their diversification and before the divergence of the Rhodophyta and Chlorophyta from the ancestral chloroplast (Durnford et al. 1999). A truncation of the Vio cycle was observed in the Prasinophycean alga *Mantoniella squamata* Manton and Parke (Goss et al. 1998). In this primitive green alga, the Vio cycle is limited to the Vio-Ant interconversions because of an extremely slow second de-epoxidation step from Ant to Zea due to a reduced affinity of *Mantoniella* VDE for Ant and a much faster epoxidation rate in the algae than in higher plants (Frommolt et al. 2001). This implies that the role of Zea in qE, the pH or energy component of NPQ (Müller et al. 2001) can be replaced by Ant in this alga. Formation of Zea can, however, be obtained by artificially low-pH treatment (Goss et al. 1998).

Lut-epoxide is widespread among photosynthetic organisms and plant tissues. Its absence in algae discards an ancestral origin. Furthermore, it seems that Lut-epoxide concentration is not phylogenetically determined and would depend on ecological constraints as no correlation was found between its presence and recurrent mutations in ZEP (Esteban et al. 2009). Altogether, the xanthophyll cycle operation seems involved in nearly all photosynthetic eukaryotes (terrestrial and marine) in the photoprotection of chloroplast membranes, with the possible exception of the Cryptophyceae class of algae (Stransky and Hager 1970; Rmiki et al. 1999, Ladygin 2008).

#### 16.4.6 THE XANTHOPHYLL CYCLE: THE MECHANISTIC OF THE CYCLE AND ITS IMPLICATIONS IN THE CHLOROPLAST PHYSIOLOGY

The lumen pH is higher than 7.0 and VDE is supposed to be mobile within this compartment (Hager and Holocher 1994). When the light absorption by LHC exceeds both the possibility for its utilization by the transducers and the capacity of energy dissipation mechanisms, lumen acidification takes place. When the pH value is below 6.5, VDE binds to the thylakoid membrane and is activated (Sierfermann and Yamamoto 1975b, Hager and Holocher 1994). The process occurs in a highly cooperative manner, the pH dependence of Vio de-epoxidation exhibiting a cooperativity for protons of about 5.5 and an inflexion point around pH 6 (Pfündel and Dilley 1993, Jahns and Heyde 1999; for a review, see Horton et al. 2008). VDE activity is also regulated by the proton concentration. The highest activity has been observed for pH values lower than 5.8. The acidity also controls the equilibrium between the basic and acidic forms of the cosubstrate ascorbate (Bratt et al. 1995, Eskling et al. 1997). Anyway, the lumen pH cannot fall below 5, otherwise the electron transport at the cytochrome *b<sub>6</sub>f* complex is inhibited (Horton et al. 2008).

Once Ant produced, VDE transfers an electron and a proton from ascorbate to the epoxy group located at the other side of Ant molecule, creating Zea, water, and dehydroascorbate. This implies that the xanthophyll cycle includes a flip-flop movement of the monoepoxidated xanthophyll in the thylakoid membrane. Altogether, the kinetics of the de-epoxidation is likely to be limited by the xanthophyll diffusion within the membrane (Macko et al. 2002). Indeed, the sorting of the Vio molecules from the proteins and their diffusion within the lipid phase to VDE for conversion to Ant exhibit a four to six times higher rate constant than the conversion of Ant to Zea (Yamamoto and Higashi 1978, Härtel et al. 1996), and thus represents the rate-limiting step of de-epoxidation (for a review, see Jahns et al. 2009).

Despite the fact that the expression of the gene encoding VDE has been shown to vary in response to light intensity (Bugos et al. 1999), the control of VDE activity through the modification of the gene expression seems minor (Macko et al. 2002, Deng et al. 2003).

The regulation of the epoxidation reactions is limited and does not involve a control by the stromal pH (Takeguchi and Yamamoto 1968, Siefermann and Yamamoto 1975a). ZEP can be downregulated after exposure of plants to high light (Jahns and Mische 1996, Verhoeven et al. 1996, Reinhold et al. 2008) or under overwintering conditions (Öquist and Huner 2003). The biochemical basis of the regulation is so far unclear but would involve ZEP phosphorylation (Xu et al. 1999; for a review, see Jahns et al. 2009).

A linear relationship has been found between the amount of Zea produced and the capacity for energy dissipation. This dissipation is reflected in the NPQ. NPQ is formed by several components and is therefore kinetically heterogeneous (Horton and Hague 1988). In moderate excess light, that is, in absence of accumulated damaged PSII reaction centers, NPQ is found to form and relax with two major components, reflecting events taking place in the antenna of PSII. These two components are qE (Müller et al. 2001) and the slowly reversible component of NPQ, namely, qI (Jahns and Mische 1996, Verhoeven et al. 1996).

In the mutant *npq1*, which is defective in VDE, most of qE is inhibited (Niyogi et al. 1998), while the double mutant *npq1 lut2* (the mutant *lut2* is unable to synthesize Lut (Pogson et al. 1998)) is devoid in qE, suggesting a role for Lut in qE (Niyogi et al. 2001) or that alteration in the Lut content could indirectly affect qE by disturbing the assembly and structure of the PSII antenna (Niyogi et al. 1997, Pogson et al. 1998, Lokstein et al. 2002). Thus, the Zea molecules produced through the xanthophyll cycle play a crucial role in the NPQ efficiency.

Besides the roles of the xanthophyll cycle in the qE component of NPQ, there are evidences for the involvement of Zea in (i) the photoprotection of the photosynthetic membranes toward stress-induced peroxidations (Havaux and Niyogi 1999, Havaux et al. 2000, Baroli et al. 2003, Havaux et al. 2007, Johnson et al. 2007). Indeed, it was demonstrated that photoinduced peroxidative damage in leaves is highly increased if the Vio cycle operation is inhibited by the addition of dithiothreitol (Sarry et al. 1994); (ii) the control of thylakoid membrane fluidity because a light-induced decrease in membrane fluidity occurs in thylakoid membranes (Gruszecki and Strzalka 1991, Havaux and Gruszecki 1993) when Vio is allowed to convert in Zea under stress, and thus the thermostability of the thylakoid membranes increases (for a review, see Gruszecki and Strzalka 2005). The LHCII prepared from illuminated leaves was shown to be poorer in xanthophyll cycle pigments than the LHCII prepared from dark-adapted leaves (Havaux and Gruszecki 1993). A diffusive displacement of Zea from the Chl–protein (CP) complexes to the surrounding lipid domain (Gruszecki 1995, Havaux and Tardy 1995) has been suggested to increase membrane rigidity; (iii) the regulation of the cyclic electron flow around PSII. In fact, when the Vio content is high, the cyclic flow of electrons around PSII is reduced (Gruszecki et al. 1995); (iv) the removal of active oxygen species formed during a stress through Zea epoxidation (Lichtenthaler and Schindler 1992, Schubert et al. 1994). The Vio formed could be again de-epoxidated. In such a case, the epoxidation could be nonenzymatic (Schubert et al. 1994).

Altogether, the operation of the xanthophyll cycle is strongly dependent on the antenna, the properties of the thylakoids, and on an active Car biosynthetic pathway. These aspects are detailed in the next paragraphs.

#### 16.4.6.1 Role of Antenna Proteins

When the proteins of the photosynthetic apparatus are separated by nondenaturing electrophoresis, Vio is found as a rather high proportion in the free pigment fraction, indicating that Vio is very weakly bound to these proteins (Lee and Thornber 1995). On the other hand, Vio is present at the periphery of the LHC (Kühlbrandt et al. 1994). Thus, the lumen acidification would favor Vio liberation from Vio-binding site in the different peripheral antenna proteins. Thus, the peripheral and weak binding of Vio are therefore two conditions allowing its involvement in the xanthophyll cycle (Demmig-Adams and Adams 1996), whereas Zea would have to return into the LHC to quench the energy (for a review, see Havaux 1998).

The resolution of the crystal structure of LHCII from spinach and pea and the discovery of Vio binding to it (Liu et al. 2004) allowed to get more information on the participation of pigment–protein

complexes in NPQ. Most evidence is given that PSII is dimeric in the appressed grana parts of the thylakoid membranes of green plants. This aggregated state may be stabilized by protein phosphorylation (Kruse et al. 1997), binding with phosphatidylglycerol (Kruse et al. 2000) and with extrinsic proteins (Boekema et al. 2000). PSII antennae associated with dimeric PSII core complexes constitute PSII-LHCII supercomplexes containing two to four copies of trimeric LHCII complexes (LHcb1–3 proteins) per PSII core dimer, together with three “minor” antenna complexes: LHcb4 (CP29), LHcb5 (CP26), and LHcb6 (CP24). Each protein binds at least one Vio molecule in the dark-adapted state (for a review, Dekker and Boekema 2005). Most of the Vio molecules are bound by the trimeric antenna complex LHCII at the peripheral Vio1 site (Liu et al. 2004), while the “minor” monomeric complexes CP29, CP26, and CP24 bind one to two Vio molecules (Ruban et al. 2002), one of which may be at the internal Lut2 site that binds Lut in LHCII (Morosinotto et al. 2002, Wehner et al. 2006). Upon exposure to excess light, VDE leads to the appearance of Ant and Zea. Vio binding from site Vio1 is pH dependent and it is released to provide a source of substrate for VDE. Vio is also liberated from the minor complexes providing empty Car-binding sites for newly formed Zea (Morosinotto et al. 2003). The liberated Vio molecules are dissolved in the thylakoid membrane lipids. Their de-epoxidation by the lumenal enzyme VDE needs the binding of VDE to the thylakoid membranes to provide access to its substrate. Monomeric antennae, especially CP26 and CP24, show high binding rates and these proteins are probably one of the sites where Zea plays its function (Morosinotto et al. 2003). Some Zea binding to the peripheral LHCI antenna of PSI was also observed (Morosinotto et al. 2002), suggesting a PSI response to the environmental stress conditions.

#### 16.4.6.2 Change of Light Harvesting Complex Properties

The detailed relationship between qE and the operation of the xanthophyll cycle is still unknown. Actually, the biophysical mechanism of energy quenching in qE remains controversial because two mechanisms, although nonexclusive, have been proposed. The first model implies a direct role of Zea in the antenna via the formation of a quenching complex with a short fluorescence lifetime (Gilmore et al. 1995). Zea operates in the quenching through a charge-transfer mechanism (Holt et al. 2005) in the minor LHC complexes associated with PSII, that is, CP29 (Lhcb4), CP26 (Lhcb5), and CP24 (Lhcb6) (Ahn et al. 2008, Avenson et al. 2008, 2009). The charge-transfer mechanism involves an energy transfer from closely coupled Chl and Zea molecules that transiently produce pigment ions, a Chl anion and a Zea cation, respectively (Frank et al. 1994). The subsequent charge recombination dissipates the excitation energy as heat.

In the second model, Zea is not required for qE (Rees et al. 1989, Horton et al. 1996, Havir et al. 1997) but is involved as an indirect allosteric regulator (Crouchman et al. 2006, for a review, see Horton et al. 2008) of the pH sensitivity of qE (Noctor et al. 1991). In this model, qE arises from a conformational change (Horton et al. 1991) in the peripheral, trimeric antenna of PSII (Pascal et al. 2005) and its mechanism involves an energy transfer from Chl to Lut1 (Ruban et al. 2007). Evidences that these events are of a cooperative nature, that is, involving interactions between subunits of PSII antenna have been presented (Noctor et al. 1991, Ruban et al. 2001). The interactions between LHCII proteins are influenced by divalent cations. The influence of the changes in their concentration of the curve qE *versus* lumen pH supports the cooperative nature of qE (Noctor et al. 1993). According to the model, the LHCII can exist under four states (Horton et al. 1991; for a review, see Horton et al. 2008). During the transition State I to State III, the lumen acidifies rapidly and LHC proteins are protonated. The intensity of the lumen acidification is, however, insufficient to saturate qE. The transition State III and State IV occurs while VDE takes place and the qE slowly builds up. When the light source is turned off, qE relaxes following the collapse of the  $\Delta$ pH. If the light source is again switched on, qE increases very rapidly to its maximum capacity because the Zea molecules formed during the previous illumination keep the photosynthetic apparatus “light activated”. In this case, the system shifts rapidly from State II to State IV, allowing photosynthesis and energy dissipation through qE to function at its maximal rates (Horton et al. 1991; for reviews,

see Horton et al. 1996, Horton et al. 2008). Using the double mutant *npq1 lut2*, Li et al. (2009) obtained evidences for the first mechanism and also for the involvement of Lut in qE.

Among the additional factors involved in both mechanisms, PsbS, a small Lhc-like protein, was shown to have a key role in the dissipation of excess energy in higher plants (Li et al. 2000) and there is evidence that its level is correlated with the extent of NPQ (Li et al. 2002). Upon lowering of the luminal pH, a rapid response, prior to xanthophyll cycle operation, called quenching 1 by Morosinotto et al. (2003) was shown to involve protonation of luminal exposed acidic residues of PsbS. More recently, PsbS has been proposed to form a quenching complex together with LHCII and Zea (Bonente et al. 2008). Thus, PsbS would act as a sensor of lumen acidification that triggers conformational changes in the PSII antenna such as LHC aggregation (Garab et al. 1988, Croft and Yerkes 1994, Horton et al. 1994, Ruban and Horton 1994, Walters et al. 1994, Ruban et al. 1997), triggering a more efficient Chl deexcitation (Ahn et al. 2008, Avenson et al. 2008, Horton et al. 2008). The aggregation may be stimulated by the addition of Zea and inhibited by Vio (Ruban et al. 1997). The action of the pigments would be mediated by their difference in hydrophobicity, that is, Zea is much more hydrophobic than Vio (Ruban et al. 1993, Darko et al. 2000). This would fit with the fact that the transmembrane organization of the xanthophyll cycle, with VDE in the thylakoid lumen and ZEP located on the stromal side, implies a certain freedom of movement of the xanthophyll cycle pigments in the membrane lipid to allow contact between the enzymes and the head group of the xanthophylls. A role for Elips and Ohps, two groups of PsbS-related stress-induced proteins, was suggested in the xanthophyll cycle-associated photoprotection (Demmig-Adams et al. 2006, Zarter et al. 2006).

#### 16.4.6.3 Role for Membrane Lipids

Activity *in vitro* of VDE was shown to require MGDG, the main lipid of thylakoid membranes (Yamamoto and Higashi 1978), and the essential role of this lipid for VDE activity in liposomes was further confirmed more recently (Goss et al. 2006). Because Vio molecules are liberated in the lipid phase of membranes, VDE needs to bind to the membrane for its activity. It was proposed that VDE would attach to MGDG-enriched membrane domains where de-epoxidation would occur (Latowski et al. 2002). In liposomes composed of phosphatidylcholine and MGDG, as in native thylakoid membranes, inverted hexagonal structures ( $H_{II}$ ) have been described (Quinn and Williams, 1983). In such liposomes, VDE activity depends on the rate of lateral diffusion to MGDG domains and thus on the MGDG/Vio ratio (Latowski et al. 2002). The presence of ( $H_{II}$ ) phases in the MGDG domains facilitates the flip-flop movement of the Ant molecule to allow VDE access to the second epoxy group and conversion of Ant to Zea. An updated model for the mechanism of xanthophyll conversions was recently proposed by Jahns et al. (2009). In this model, the presence of  $H_{II}$  domains and Vio molecules free in MGDG phases are required. They also propose that the transition to the light-adapted state is accompanied by aggregation of LHCII complexes, which facilitates the liberation of Vio molecules from the external VioI-binding sites. After binding of VDE to the MGDG phases, Vio is de-epoxidized into Zea. The decreased solubility of Zea in MGDG compared with phosphatidylcholine facilitates reentrance of Zea in the bilayer phase of the membranes where the antenna proteins are located. The binding of Zea to the minor antenna proteins of PSII (Lhcb4–6) could facilitate the dissipation of energy *via* a Zea cation radical.

#### 16.4.6.4 Xanthophyll Cycle Pool of Pigments Is Dynamic

So far, we described the xanthophyll cycle as a device catalyzing pigment interconversions. There are more and more results suggesting that, in fact, the amount of pigments in the xanthophyll cycle pool depends on the environmental conditions but is also adjusted quantitatively as a function of the stress intensity (Esteban et al. 2007). For instance, in the two Pheophyceae, *Pelvetia caniculata* and *Laminaria saccharina*, the amounts of Zea accumulated through the operation of the xanthophyll cycle operation after a light stress are very different on a Chl *a* basis. Such a difference has been shown to be one of the main factors responsible for the specific distributions of these two species at opposite levels on the seashore (Harker et al. 1999). Duckweed (*Lemna minor*) plants grown under

high light present a higher xanthophyll cycle pool of pigments and also a higher proportion of transformable Vio than the plants grown under low light. When plants were transferred to a high-light environment, a quick adjustment of the Car composition was observed. It mostly concerns the pigments involved in the xanthophyll cycle. This increase in pigments results of a *de novo* synthesis of Cars because in the presence of norflurazon, an inhibitor of carotenogenesis, this increase is inhibited. The extent of this light-induced synthesis is proportional to the light treatment and also on the operation of the xanthophyll cycle. Accordingly, the inhibition of the xanthophyll cycle by dithiothreitol also abolished the light-induced carotenogenesis (Garcia-Plazaola et al. 2002b).

## 16.5 SECONDARY CAROTENOID AND APOCAROTENOID BIOSYNTHESIS IN ALGAE AND HIGHER PLANTS

Cars are found in all photosynthetic organisms and in some bacteria and fungi (for a review, see Almeida and Cerda-Olmedo 2008, Lemoine et al. 2008). While primary Cars constitute functional components of the photosynthetic apparatus, the secondary Cars are accumulated in oil droplets in the cytoplasm or in the plastids.

Car synthesis has been investigated since the mid-1960s and the genes for almost all the enzymes involved in this biosynthesis have been cloned and analyzed in various organisms (Ye et al. 2008). Two distinct pathways may produce the isoprenoid precursor, isopentenyl diphosphate (IPP): the cytosolic mevalonate pathway and plastidic nonmevalonate or methylerythritol 4-phosphate (MEP) pathway (Das et al. 2007). The MEP pathway seems to be the favored way to synthesize Cars in higher plants (DellaPenna and Pogson 2006) and the acetate/mevalonate pathway was shown to be absent in chlorophytes (Schwender et al. 2001). The condensation of glyceraldehyde-3-phosphate and pyruvate gives the 5-carbon compound IPP and its isomer dimethylallyl pyrophosphate. A series of condensation reactions of one dimethylallyl pyrophosphate molecule and three IPP molecules gives the 20-carbon geranyl diphosphate, which produce the 40-carbon Car precursor phytoene. Successive desaturation reactions catalyzed by phytoene desaturase and  $\zeta$ -carotene desaturase yield lycopene. Lycopene is cyclized into  $\beta$ -carotene, a nonoxygenated Car, by the lycopene  $\beta$ -cyclase (LCY-B). The oxygenated derivatives of carotenes are designated as xanthophylls—the key components of the LHCs (DellaPenna and Pogson 2006). The Car metabolism is not limited to the chloroplast in photosynthetic organisms since  $\beta$ -carotene oxidation into astaxanthin occurs in the cytosol of *Haematococcus pluvialis*, and it has been shown in *Marchantia polymorpha* that the complete synthesis of Cars occurs in cytoplasmic oil bodies (Suire et al. 2000). Xanthophylls can also be metabolized further into Apocars.

### 16.5.1 SECONDARY CAROTENOIDS IN MICROALGAE

Despite the fact that several green microalgal species such as *H. pluvialis*, *Dunaliella* sp., *Neochloris wimmeri*, *Scenedesmus vacuolatus*, *Scotiellopsis oocystiformis*, *Chlorella zofingiensis*, and *Protosiphon botryoides* are able to accumulate secondary Cars under stressful environmental conditions (Kopecky et al. 2000, Orosa et al. 2000), most of the studies have been performed on *Dunaliella* sp. and *H. pluvialis*, probably because these two taxons are used for the commercial production of Cars (e.g., Zhang et al. 1999, Das et al. 2007).

#### 16.5.1.1 *Dunaliella* sp.: A Model for the Study of $\beta$ -Carotene Biosynthesis

*Dunaliella salina*, a halotolerant alga, accumulates large amounts of  $\beta$ -carotene (up to 10% of the dry algal biomass) under high light intensity, high salinity, high temperature, heavy metal stress, and/or insufficient nitrogen and phosphate conditions (Ramos et al. 2008). The other Cars synthesized are  $\alpha$ -carotene, Lut, Zea, cryptoxanthin, and neoxanthin. The cell color of *Dunaliella* changed from green to orange-red following Car accumulation (Lamers et al. 2008). Because *D. salina* accumulates high amounts of  $\beta$ -carotene under stress conditions, the culture of this alga

is the most important process for natural production of this Car. This alga thus represents a useful model to understand the mechanism of Car formation (Ye et al. 2008). Enzymes and intermediates in the nonmevalonate pathway are seldomly analyzed, but more information would be obtained after the release of the genome sequence now under sequencing on the DOE Joint Genome Institute Web site (<http://www.jgi.doe.gov/>). In biflagellate and motile cells and especially under carotenogenic conditions, the  $\beta$ -carotene was accumulated as lipid globules in the interthylakoid spaces of the cell's single chloroplast. This membrane-free lipid globule exclusively contained more than half of  $\beta$ -carotene, neutral lipids, and a small amount of a protein (38 kDa) called the carotene globule protein that could stabilize the lipid globule within the chloroplast (Hadi et al. 2008, Ye et al. 2008).

Car synthesis in *Dunaliella* has recently been shown to proceed *via* the MEP pathway (Caparobles et al. 2009). Key sequences of the carotenogenesis production were isolated from a few species of *Dunaliella* taxon and sequenced: 1-deoxyxylulose-5-phosphate synthase (DXS), responsible of the first step in the biosynthesis of IPP, phytoene synthase, phytoene desaturase, and LYC, which catalyzes cyclization at both ends of the linear lycopene to form cyclic  $\beta$ -carotene (Yan et al. 2005, Sanchez-Estudillo et al. 2006, Zhu et al. 2008).

During environmental stress, the generation of ROS, which damage proteins, carbohydrates, and DNA, triggers the accumulation of internal antioxidants such as  $\beta$ -carotene in *Dunaliella* (Murthy et al. 2005, Ye et al. 2008). The 9-*cis* stereoisomer is more efficient than all-*trans*- $\beta$ -carotene in scavenging of ROS (Ye et al. 2008). These properties of the Cars explained in part why the medical science used these molecules against many kinds of human diseases (Handelman 2001, Higuera-Ciapara et al. 2006).

Triacylglycerol biosynthesis and the carotenogenesis were shown to be interdependent in *Dunaliella* (Rabbani et al. 1998), newly formed lipid droplets being necessary for the accumulation of  $\beta$ -carotene. Lipid metabolism is also affected by the increase of the volume of *Dunaliella* cells because they are surrounded by a thin elastic membrane, and the membrane composition changes under stress conditions (Murthy et al. 2005, Ye et al. 2008).

#### 16.5.1.2 *Haematococcus pluvialis*: A Model to Study Astaxanthin Biosynthesis

The main factors inducing astaxanthin accumulation in *H. pluvialis* are low nitrate, high light, and high temperature (Lemoine et al. 2008). Several genes, such those coding IPP isomerase, phytoene synthase (PSY), phytoene desaturase (PDS), lycopene  $\beta$ -cyclase (LCY-B), and  $\beta$ -carotene ketolase, involved in the Car biosynthesis are activated under stress conditions.

Astaxanthin biosynthesis is the most studied secondary Car and is produced by  $\alpha$ -proteobacteria, fungi, and plants (Tao et al. 2006, Martin et al. 2008). Astaxanthin is synthesized from  $\beta$ -carotene but the biosynthesis pathways in bacteria, fungi, algae, and plants differ (Martin et al. 2008). In *H. pluvialis*, the first astaxanthin molecules are synthesized at the expense of the  $\beta$ -carotene molecules taken from the photosynthetic apparatus. To sustain the large accumulation of astaxanthin molecules, a *de novo*  $\beta$ -carotene synthesis is triggered when the  $\beta$ -carotene pool, present before the stress, is almost exhausted (Schoefs et al. 2001). Regardless of their origin, the  $\beta$ -carotene molecules are exported in the cytoplasm in lipid vesicles where they are transformed into astaxanthin that are, in turn, esterified with fatty acid molecules (for a review, see Lemoine et al. 2008). The astaxanthin biosynthesis route is now quite well defined:  $\beta$ -carotene is transformed into echinenone after ketolation with either canthaxanthine or hydroxy-echinenone as a first intermediate and further hydroxylation of adonirubin (Schoefs et al. 2001). The use of a pharmacological approach has shown that the other routes *via* adonixanthin are poorly performed (Fraser et al. 1998; Hirschberg 1998, Schoefs et al. 2001). There is at least a  $\beta$ -carotene hydrolase belonging to the cytochrome P-450 monooxygenase protein superfamily that is involved in this pathway (Schoefs et al. 2001). In the fungus *Xanthophyllomyces dendrorhous*, the conversion of  $\beta$ -carotene into astaxanthin could be catalyzed by a single multifunction  $\beta$ -carotene oxidase (Ojima et al. 2006) by the combined activity of a  $\beta$ -carotene hydroxylase and a  $\beta$ -carotene ketolase working successively on different intermediates (Martin et al. 2008).

Some Car could be produced through chemosynthetic pathway but they are devoid of stereoisomers. To produce Cars, researchers used engineered microorganisms such as *E. coli* and yeast or transgenic plants such as *Oryza sativa* employing transformation with carotenogenic genes (Ye et al. 2008). The carotenogenesis genes of *D. salina* and *H. pluvialis* may promote massive accumulation of Cars in heterologous expression systems. The genome of *D. salina* could be used as a tool to identify and find the sequences of enzymes such as the isomerase involved in the formation of Cars with the active configuration.

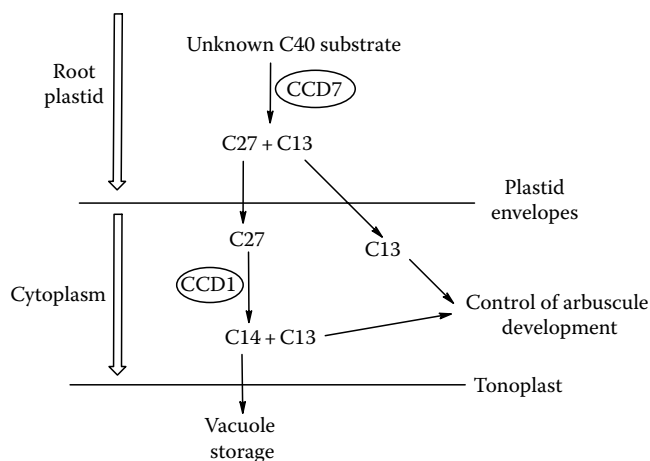
### 16.5.2 ROOT COLONIZATION BY ARBUSCULAR MYCORRHIZAL FUNGI TRIGGERS THE PRODUCTION OF SECONDARY CAROTENOIDS AND APOCAROTENOIDS

Most of land plants form mutualistic association or symbiosis with soil-borne fungi. The arbuscular mycorrhizal symbiosis is the most frequently encountered since it concerns *ca.* 80% of the plants and 90% of crop plants. In arbuscular mycorrhizal symbiosis, the fungus colonizes root cortical cells forming highly branched structures, the so-called arbuscule, that facilitates the uptake of the soil nutrients such as phosphate, nitrate, or water, while the plant partner provides the fungus with photosynthetically synthesized carbohydrates (up 20% of plant-fixed carbon) (Parniske 2008). The formation of the arbuscules leads to a complex and profound reorganization of the subcellular structures of the cortical cells including a massive proliferation of the plastids of the colonized root cortical cells. These plastids are connected to each other by stromules (Köhler et al. 1997, Tobin 1997; for a review, see Fester 2008), forming a dynamic network that is formed and degraded with the formation and the senescence of the arbuscules (Fester et al. 2001, Strack and Fester 2006; for reviews, see Bonfante-Fasolo 1984, Gianinazzi-Pearson 1996). In root plastids, different sets of enzymes catalyze numerous biochemical processes such as those leading to the production of fatty acids, amino acids, purines and pyrimidines, nitrogen assimilation, and isoprenoids (Miernyk 1985, Daher et al. 2010). The activities of these pathways are enhanced during mycorrhization suggesting a crucial role for the plastids in the accumulation of Apocars and during the mycorrhization (Fester et al. 2001, 2007, Lohse et al. 2005). In particular, the cellular program driving the colonization contains lines specifying reorientation of the root plastid metabolism to the production of secondary metabolites including Cars and Apocars to a point that the white roots become yellow when colonized (Klingner et al. 1995, Fester et al. 2002a, Schliemann et al. 2006). The core structure of the yellow pigment contains an acyclic C14 polyene, namely, mycorradicin. This pigment is accompanied by a C13 cyclohexenone diglucoside (Fester et al. 2002a, Walter et al. 2007). Both Apocar types are actually present as mixture because they are differently esterified (Maier et al. 1995, Fester et al. 2002a) and their production results of the oxidative cleavage of a common Car precursor (Walter et al. 2000). The Apocars are massively deposited in the vacuole (Klingner et al. 1995) and probably in the cytoplasm too (Fester et al. 2002a). The accumulation of the yellow pigments mostly occur during the late phase of mycorrhization, that is, during arbuscule degradation, phase of the mycorrhization process during which the plastid network is disintegrated (Fester et al. 2001).

#### 16.5.2.1 Biosynthesis of Apocarotenoids

The activation of the AM-dependent MEP pathway has been demonstrated for *Medicago truncatula* (Lohse et al. 2005, Floss et al. 2008a). Northern blot experiments showed an increase of the transcript levels of the DXS2 and 1-deoxy-xylulose-5-phosphate reductoisomerase, the first enzymes of the plastids-located MEP pathway, in AM roots from wheat, maize, rice, and barley (Fester et al. 2007, Walter et al. 2000, Walter et al. 2000, 2002, 2007). Transcript levels of the genes coding the enzymes involved in the Car pathway such as PDS and  $\zeta$ -carotene desaturase increase in the AM roots of *Nicotiana tabacum* (Strack and Fester 2006) but no Car accumulates because they are further metabolized into Apocars through the action of Car cleavage dioxygenase (CCD) enzymes, the production of which is induced by colonization by AM fungi (Strack and Fester 2006).





**FIGURE 16.2** Scheme presenting the biochemical pathways of mycorradicine (C14) and cyclohexenone (C13) in response of mycorrhization.

CCD activities exhibit a high degree of regiospecificity and stereospecificity. They give rise to a variety of products with different biological functions as Apocar aromatic volatiles of flower scents, fruit aroma, phytohormones such as abscissic acid, and strigolactone (Akiyama 2007, Garcia-Garrido et al. 2009). Using a very elegant approach, Floss et al. (2008a) demonstrated that the CCD enzyme that uses the C40 Car precursor is CCD7. It has been shown that CCD7 cleaves Cars asymmetrically, producing C13 and C27 fragments (Bouvier et al. 2003, Rubio et al. 2008) (Figure 16.2). Another candidate for the catalysis of the reaction would be CCD4, but expression data have shown that this enzyme is not produced in roots (Ohmiya et al. 2006, Floss et al. 2008a, Rubio et al. 2008). This reaction takes place in the plastids, and the products are exported to the cytoplasm where they are further metabolized by the CCD1 enzyme into C13 cyclohexenone and C14 mycorradicin (Floss et al. 2008a, Vogel et al. 2008) (Figure 16.2). This scheme fits with the subcellular localization of the enzymatic activities. CCD1 that is able to directly split up a C40 into C13 and C14 (Schwartz et al. 2001) is the only CCD to be localized outside plastids (Bouvier et al. 2003, Tan et al. 2003, Aldridge et al. 2006) and, consequently, it has no access to the C40 precursor. Thus, it is its second catalytic activity (cleavage at the 5,6/5',6' positions) that is at work in the cytoplasm (Figure 16.2). These results highlight that, to really understand the functioning of a biochemical process such as the production of Apocars, it is crucial to consider the subcellular localization(s) of the enzymatic activities (for reviews, see Schoefs 2008, Seddas et al. 2009).

The Car fragments are partly esterified and deposited as small lipophilic droplets in the vacuoles of root cortical cells (Strack and Fester 2006). Traces of  $\zeta$ -carotene were detected and no accumulation of other detectable intermediates suggested that the biosynthesis of the accumulating Apocars appeared to proceed rapidly (Fester et al. 2007). Very importantly, the accumulation of these secondary Apocars is restricted to the colonized cells because the expression of the *DXS2* gene increases only in these cells (Floss et al. 2008b), reinforcing the idea that plastids play a crucial role in the metabolism of these cells.

Another type of secondary Apocars, such as the strigolactone family of molecules, is produced by plant roots (Akiyama 2007, Garcia-Garrido et al. 2009). Their synthesis would also involve CCD7 but in this case, the C27 product would be cleaved into a C9 and a C18 products through the catalytic action of CCD8 (Schwartz et al. 2004; Alder et al. 2008) (Figure 16.2).

### 16.5.2.2 Roles and Functions of Secondary Apocarotenoids

Strigolactones, a novel class of plant hormone, are released usually in trace amounts from the roots on phosphate deficiency but in sufficient amount to be detected by new potential hosts (Strack and Fester 2006, Garcia-Garrido et al. 2009, Seddas et al. 2009). Strigolactone stimulates spore

germination in some AM fungi (Parniske 2008) and induces a continued hyphal growth and a profuse branching of hyphae, and could also activate the respiration of the arbuscular mycorrhizal fungi (for a review, see Seddas et al. 2009).

Abscissic acid, another plant hormone, is an Apocar. A possible role of the abscissic acid in AM roots was suggested because the level of this phytohormone increases during the fungal symbiosis in *Zea mays* and *Glycine max* (Strack and Fester 2006, Parniske 2008, Seddas et al. 2009).

The accumulated Apocars C13 and C14 may have structural functions and could provide efficient protection against oxidative stress (ROS scavenging) as the general antioxidant function of Cars accumulated under environmental stress (Fester et al. 2002b, Floss et al. 2008a). ROS could act as second messengers inducing the Car biosynthesis and/or are required for signaling fungal cell death (Strack and Fester 2006). Mycorradicin might play a role in the detoxification of  $H_2O_2$  generated during the arbuscule disintegration. Apocars may protect membranes from oxidative damage, limiting oxygen penetration to the hydrophobic membrane core (Strack and Fester 2006).

In plants having *CCD1* gene silenced, the amount of C13 cyclohexenone decreased strongly, whereas the amount of senescent or dead arbuscules increased (Floss et al. 2008b). Therefore, it was proposed that cyclohexenone is involved in the control of the duration of the functionality of arbuscules (Walter et al. 2007, Floss et al. 2008b). This conclusion fits with the fact that exogenous blumenin shows a negative influence on AM (Fester et al. 1999, Walter et al. 2007). In contrast, cyclohexenone derivatives (other C13) were not responsible for the systemic suppression of mycorrhization in pre-colonized barley plants (Vierheilig et al. 2000), and mycorradicin did not suppress an elicitor-induced oxidative burst reaction in *N. tabacum* and *Medicago sativa* cell cultures (Schröder et al. 2001).

## CONCLUSIONS AND PERSPECTIVES

Cars and Apocars are commonly concerned in biotic and abiotic stresses. They participate efficiently to the various plant responses and in these sense, they constitute a crucial element of the plant survival strategies. An essential function of Cars is to prevent the chloroplasts against harmful photooxidative reactions either through a direct dissipation of the excess of energy as heat or by participating in the formation of the NPQ through their involvement in the xanthophyll cycle. Besides these contributions to plant physiology, Cars and Apocars act as storage compounds, allowing stressed algae to overcome the disruption of the photosynthetic activity triggered by the stress. Many progresses that have been performed in the elucidation of the metabolic pathways leading to the production of the Cars, and physiological studies have suggested that previously described catabolic pathways, might actually be metabolic and/or regulation pathways. Thus, they do not solely correspond to degradation of useless molecules but they could produce crucial actors of plants physiology such as strigolactones and cyclohexenones. This has opened completely new avenues for research in different fields of plant sciences. Altogether, the central role of different types of plastids (chloroplasts and root plastids) in plant life is strongly reinforced.

## ABBREVIATIONS

|        |                                  |
|--------|----------------------------------|
| AMS    | Arbuscular mycorrhizal symbiosis |
| Ant    | Antheraxanthin                   |
| Apocar | Apocarotenoid                    |
| Car    | Carotenoid                       |
| CCD    | Carotenoid cleaving dioxygenase  |
| Chl    | Chlorophyll                      |
| CP     | Chlorophyll-protein              |
| Ddx    | Diadinoxanthin                   |
| DDE    | Diadinoxanthin de-epoxidase      |
| DEP    | Diatoxanthin epoxidase           |

|       |                                         |
|-------|-----------------------------------------|
| Dtx   | Diatoxanthin                            |
| DXS2  | 1-deoxy-D-xylulose 5-phosphate synthase |
| IPP   | Isopentenyl diphosphate                 |
| LCY   | Lycopene cyclase                        |
| Lcy-B | Lycopene $\beta$ -cyclase               |
| LHC   | Light-harvesting complex                |
| Lut   | Lutein                                  |
| MEP   | Methylerythritol 4-phosphate            |
| MVA   | Mevalonate pathway                      |
| NPQ   | Nonphotochemical quenching              |
| PDS   | Phytoene desaturase                     |
| PS    | Photosystem                             |
| PSY   | Phytoene synthase                       |
| qE    | High-energy state quenching             |
| qI    | Photoinhibition-related quenching       |
| VDE   | Violaxanthin de-epoxidase               |
| Vio   | Violaxanthin                            |
| Zea   | Zeaxanthin                              |
| ZEP   | Zea epoxidase                           |

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# 17 Thermoluminescence Study of Photosystem II Activity in Resurrection Plant *Haberlea rhodopensis* during Desiccation

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## 17.1 INTRODUCTION

Desiccation-tolerant (poikilohydric) plants represent a unique group of organisms, which are able to withstand loss of water to an air-dry state and to survive extended periods of severe water deficit [1,2]. Poikilohydry is a relatively common phenomenon in nonvascular taxa such as lichens, algae, and bryophytes, but only 74 pteridophytes and 145 angiosperms belong to these so-called resurrection plants [3]. In a desiccated state, their physiological functions, including photosynthetic activity completely ceased, but during rehydration this activity can be fully restored, with different rates in homoiochlorophyllous (HDT) and in poikilochlorophyllous (PDT) desiccation-tolerant plants [4,5].

Various aspects of the desiccation tolerance in vascular plants have received considerable attention, the latest efforts being focused on clarifying the physiological and molecular basis of this phenomenon [6–20]. However, until now the exact mechanisms which preserve the highly sensitive photosynthetic system in HDT plants during desiccation, and the characteristics of the recoverable photosynthetic system in the desiccated stage are still not well understood. The complete reconstitution of chloroplast structure and functional activity in resurrection plants on rehydration suggests some peculiarities of thylakoid membrane and/or chloroplast stroma composition [21,22], which make these plants a very suitable model system for investigation of photosystem II (PSII) complex perturbations and its adaptive plasticity in the course of desiccation and rehydration.

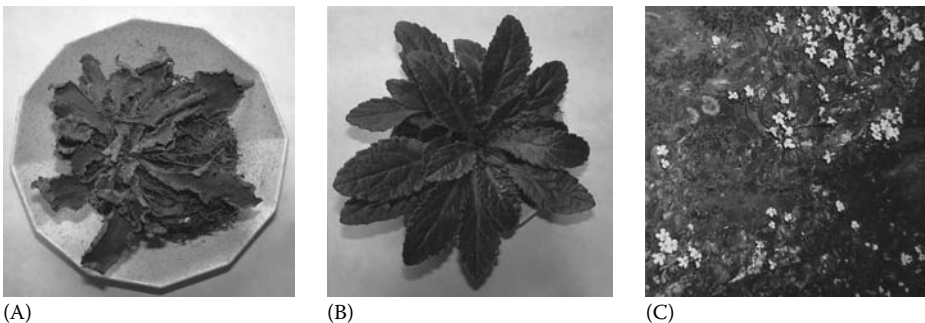
During our studies of *Haberlea rhodopensis* Friv., using a highly sensitive thermoluminescence (TL) technique, we observed some peculiarities of PSII redox reactions that can reflect some specific adaptive characteristics of the photosynthetic system, related to desiccation tolerance of this resurrection plant. In addition to multiple mechanisms for chloroplast integrity preservation, the observed stabilization of charge storage in PSII complex together with a strong reduction of the total number of PSII centers without any changes in their energy status, can explain the fast recovery of the photosynthetic activity after desiccation.

## 17.2 STRATEGIES OF RESURRECTION ANGIOSPERM *HABERLEA RHODOPENSIS* TO PRESERVE CELL INTEGRITY

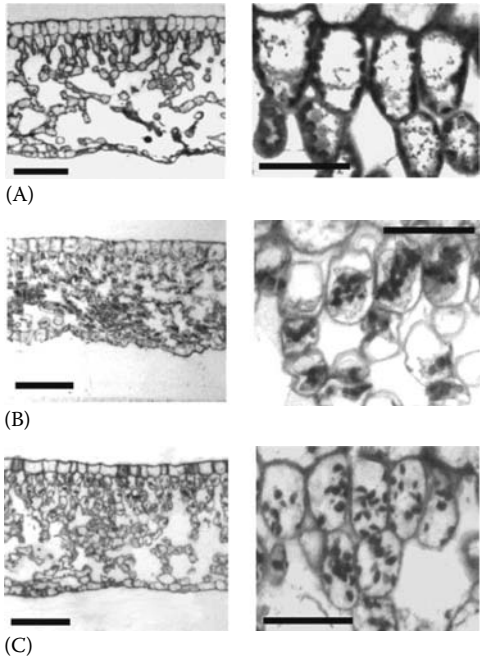
The Balkan endemic *Haberlea rhodopensis* Friv. (Gesneriaceae) is a unique species of European flora, (spread in Bulgaria and Northeastern Greece), belonging to a very small group of desiccation-tolerant vascular flowering plants (Figure 17.1C). The current distribution of the plants is restricted to the northern rocky slopes of gorges and canyons, mainly of foothills, sometimes reaching the alpine belts. Its natural habitats are characterized by high mean annual temperatures (of about 20°C), frequent mist and dew fall in the early morning hours and in the evening, and high periodic rainfalls totaling about 600 mm annually. Besides episodic droughts and high temperatures, pronounced short and long-term changes in irradiance may be the major environmental constraints affecting water balance and photosynthetic activity of these rosette plants.

Homoiochlorophyllous desiccation-tolerant (HDT) plants evolve various morphological and physiological mechanisms to preserve their intrachloroplastic membrane system and photosynthetic apparatus in an easily and rapidly recoverable form, which can be ready to function in a normal way immediately after rehydration, following the desiccated stage [5]. During drying, a controlled folding of *Haberlea rhodopensis* leaves occurs (Figure 17.1A) with the abaxial surface exposure to light thought to be an important protective strategy in HDT plants to preserve their chloroplasts from photoinhibitory damages [23–25]. Simultaneously, a phenomenon of leaf surface decrease (more than 60% compared to fully hydrated leaves), accompanied by a reduction in specific leaf area, was also observed for *H. rhodopensis* leaves, dehydrated to about 30% relative water content (RWC) [26].

These typical resurrection plant reactions, which minimize mechanical damage, are due to cell volume reduction in conformity with cell walls folding (Figure 17.2B). The lack of significant differences in the electrolyte leakage between fully hydrated, desiccated, and rehydrated *H. rhodopensis* leaves confirms the preservation of membrane integrity (Table 17.1). In contrast to desiccation-tolerant species, the observed leaf surface decrease in mesophytic spinach during desiccation was only 20%, which disturbed membrane integrity and induced irreversible injuries of the cell membranes, leading to further substantial increase in leakage upon rehydration of the leaves (Table 17.1). We also found clear differences in the level of MDA, H<sub>2</sub>O<sub>2</sub>, and proline accumulation between *H. rhodopensis* and spinach leaves, confirming the symptoms of injury in non-tolerant plants.



**FIGURE 17.1** Morphological changes in the resurrection plant *Haberlea rhodopensis* Friv. (Gesneriaceae). (A) Desiccated plant. (B) Fully hydrated plant. (C) *Haberlea rhodopensis* plants in their natural habitat.



**FIGURE 17.2** Light microscope micrograph cross-sections from detached leaves of the homoiochlorophyllous resurrection plant *Haberlea rhodopensis* treated in the dark. (A) The fully hydrated leaf shows extended oval cells with a large central vacuole and peripheral location of the chloroplasts. (B) Dehydrated to 20% RWC leaf. The cells become smaller with irregular shape, the central vacuole have shrunk or even disappeared, with a formation of several small vacuoles, the chloroplasts are scattered in the cytoplasm. (C) During rehydration for 12 h, the cells have notably restored their normal shape and plastid location. Bars = 20  $\mu\text{m}$  (left) and 100  $\mu\text{m}$  (right).

**TABLE 17.1**  
**Changes in Electrolyte Leakage, Malondialdehyde, Hydrogen Peroxide, and Proline Content in *Haberlea rhodopensis* and Spinach Leaves during Dehydration and Rehydration (R)**

| Species             | RWC (%) | Electrolyte Conductivity ( $\mu\text{S g}^{-1} \text{ DW}$ ) | Malondialdehyde ( $\mu\text{mol g}^{-1} \text{ DW}$ ) | Hydrogen Peroxide ( $\mu\text{mol g}^{-1} \text{ DW}$ ) | Proline ( $\mu\text{mol g}^{-1} \text{ DW}$ ) |
|---------------------|---------|--------------------------------------------------------------|-------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------|
| <i>Haberlea</i>     | 95      | $298 \pm 56$                                                 | $0.240 \pm 0.01$                                      | $55.75 \pm 2.38$                                        | $0.420 \pm 0.06$                              |
| <i>Haberlea</i>     | 20      | $532 \pm 25$                                                 | $0.251 \pm 0.01$                                      | $29.05 \pm 0.78$                                        | $0.543 \pm 0.04$                              |
| <i>Haberlea</i> (R) | 75      | $302 \pm 35$                                                 | $0.197 \pm 0.01$                                      | $60.13 \pm 0.85$                                        | $0.147 \pm 0.04$                              |
| Spinach             | 95      | $1076 \pm 206$                                               | $0.352 \pm 0.02$                                      | $59.50 \pm 0.40$                                        | $1.541 \pm 0.06$                              |
| Spinach             | 20      | $4490 \pm 421$                                               | $0.400 \pm 0.01$                                      | $47.63 \pm 0.38$                                        | $10.009 \pm 0.88$                             |
| Spinach (R)         | 30      | $7201 \pm 114$                                               | $0.577 \pm 0.01$                                      | $108.43 \pm 0.21$                                       | $2.794 \pm 1.10$                              |

The protection against desiccation damage in angiosperms is linked to the accumulation of carbohydrates, various compatible solutes and specific proteins as well as some changes in lipid composition. The analysis of mainly carbohydrates in the leaves of *H. rhodopensis* showed dextrans, sucrose, fructose, glucose, and starch [27]. During drying, starch content declined and large quantities of sucrose [27,28] was accumulated.

Similarly, the lipid and sterol compositions of leaves of *H. rhodopensis* changed significantly during drying [29]. The most substantial changes were observed at 50% water deficit, where linolenic



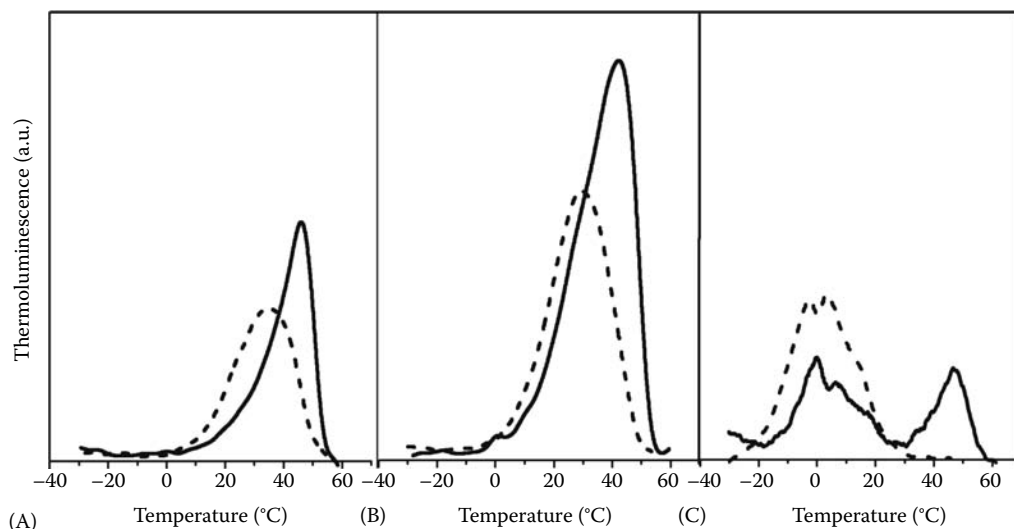
acid concentration diminished, but the campesterol/sitosterol ratio increased, causing membrane stabilization. The authors suggested that the highest proportion of DGDG (a bilayer-forming lipid) may play a role in the control of ionic permeability in the *H. rhodopensis* chloroplasts. An increased DGDG/MGDG ratio upon dehydration was also found after water stress in a tolerant variety of *Vigna unguiculata* [30], in a drought-tolerant cultivar of wheat [31], in *Ramonda serbica* [29,32], and in *Sporobolus stapfianus* [33]. Different arrangements of MGDGs and DGDGs within the thylakoid membranes [34] and changes in their proportions correlate with its physical properties.

### 17.3 FUNCTIONAL FEATURES OF PHOTOSYSTEM II IN *HABERLEA RHODOPENSIS* LEAVES STUDIED BY THERMOLUMINESCENCE

#### 17.3.1 PECULIARITIES OF THERMOLUMINESCENCE EMISSION FROM *HABERLEA RHODOPENSIS* LEAVES

*H. rhodopensis* belongs to the group of homoiochlorophyllous poikilohydric dicotyledons, which upon desiccation preserve above 80% of the chlorophyll, and its photosynthetic apparatus is able to recover very fast [35,36]. Moreover, *H. rhodopensis* has the rare ability of its resurrection occurring in detached leaves (and even a small leaf disk). Young, fully expanded leaves, from the middle of rosettes, of similar size and appearance were used in the measurements. In order to distinguish the direct effect of water loss on photosynthetic activity and to avoid photoinhibition, the dehydration of detached leaves was carried out under dark conditions. For the purpose of comparison, experiments with leaves of the desiccation-sensitive mesophytic plant *Spinacia oleraceae* L. (Chenopodiaceae) were also done.

TL glow curve parameters were used to access the functional features of PSII. TL proved to be a very sensitive and reliable biophysical method for investigation of the functioning of both PSII donor and acceptor side components (see Refs. [37–39] for review). TL signals have been assigned to result from the thermal-activated recombination of the trapped electrons and stabilized positive equivalents on the reduced quinone acceptors ( $Q_A$  or  $Q_B$ ) and on the  $S_2$  (or  $S_3$ ) oxidation state of the water-splitting complex, respectively. Figure 17.3 shows TL curves of *H. rhodopensis* leaves in comparison to those from spinach. Excitation of dark-adapted spinach leaves with a single flash (F), generating a  $S_2Q_B^-$  charge pair, induced a B band peaking at around 32°C (Figure 17.3A), which was



**FIGURE 17.3** TL from fully hydrated dark-adapted *Haberlea rhodopensis* (solid line) and spinach (dashed line) leaves excited with one (A) and two (B) saturating flashes at 5°C. (C) Leaves infiltrated with 20  $\mu$ M DCMU and excited at  $-10^{\circ}\text{C}$  with one saturating flash. Leaf disks with a diameter of 10mm were used in the experiments.

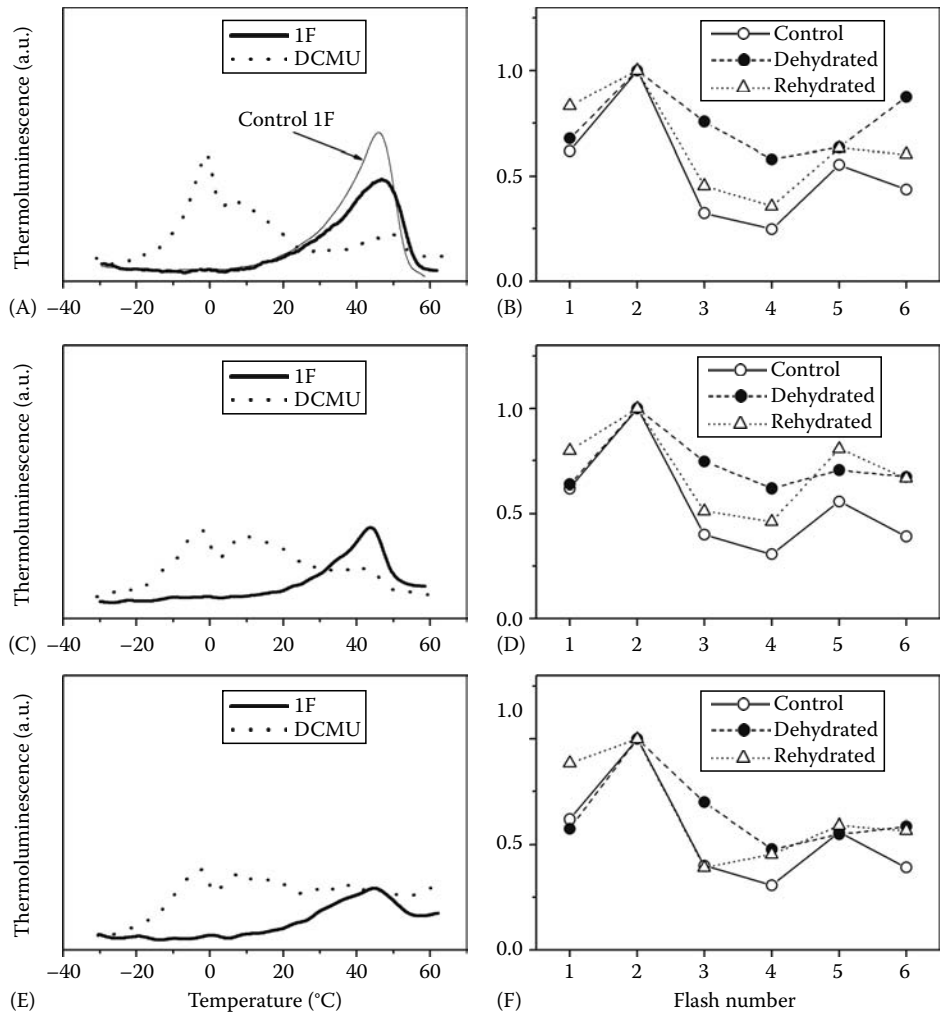
usually observed in the higher plants [40]. The most striking feature of the TL emission observed in the *H. rhodopensis* leaves was the upshift of the B-peak emission temperature to about 45°C. Similarly, different emission temperatures were registered when two ( $S_{2(3)}Q_B^-$ ) flashes were given (Figure 17.3B). The B band position at higher temperature is indicative of more stably stored  $S_{2(3)}Q_B^-$  charge pairs in the resurrection plant [41]. With intact, photosynthetically active cells, such a high TL B band emission temperature had been reported for other types of stress-tolerant organisms, namely thermophilic cyanobacterium or desiccation-tolerant ferns and lichen [22,42,43].

The high emission temperature of the TL B band from *H. rhodopensis* leaves could be attributed to some changes in the properties of redox partners on the donor or on acceptor side of PSII, or both. One way to test the contribution of  $Q_B^-$  is to monitor TL after infiltration of the leaves with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which specifically inhibits the electron transport between the primary ( $Q_A$ ) and the secondary ( $Q_B$ ) quinone acceptors. Recordings on Figure 17.3C show that DCMU treatment of spinach leaves leads to a significant downshift in B band position, concomitantly with a decrease in its amplitude and the appearance of a new, so-called Q band, peaking at around 0°C, which is thought to originate from  $S_2Q_A^-$  charge recombination [40]. In 20  $\mu$ M DCMU treated *H. rhodopensis* leaves disks the Q band also appeared at approximately the same temperature. Since the S states are the common pole for positive charges of the B and Q band, the distinct differences of B-peak temperature position in *H. rhodopensis* and spinach leaves suggest major alterations in the redox property of  $Q_B^-$  in the resurrection plant. Surprisingly, a part of B band was still clearly expressed even at higher inhibitor concentrations. These results show that some PSII reaction centers in *H. rhodopensis* leaves, with more stable stored  $S_{2(3)}Q_B^-$  charge pairs, are unsusceptible to DCMU, therefore, can possibly indicate some modifications of the redox properties of the quinone acceptor  $Q_B$  (especially in D1 core protein). In accordance with this suggestion are the data of Ohad et al. [44] and Hideg et al. [45], considering the observed incomplete suppression of the B band by DCMU after high light and UV-B irradiation as a proof for acceptor side modifications. Analogical deeper stabilization of  $S_{2(3)}Q_B^-$  charge recombination as a result of induced mutations in D1 protein have been also reported [46–49]. The already described specific lipid and sterol composition of *H. rhodopensis* leaves and the presence of different protective compounds in chloroplasts stroma may contribute to these modifications.

### 17.3.2 CHANGES IN THERMOLUMINESCENCE CHARACTERISTICS DURING DARK DESICCATION AND REHYDRATION

Under conditions of severe or prolonged water deficit, most plants are desiccation-intolerant (homoiohydric) and react to stress by the suspension of metabolism and irreversible damage to membrane structures and internal organization. Our data of changes in TL B band parameters from dehydrated spinach leaves [50] are in agreement with the respective desiccation sensitivity of this mesophytic plant. Severe dehydration of the leaves inhibits the number of operating centers, but leads predominantly to a well-expressed downshift of B band position close to Q band position. This observation is indicative for the destabilization of PSII centers as it was also shown in TL study on desiccating barley leaves [51]. It may be concluded that in mesophytic plants subjected to severe dehydration the electron transport between primary ( $Q_A$ ) and secondary ( $Q_B$ ) quinone acceptors is inhibited, and damaged oxygen-evolving complexes occur. Such PSII centers do not restore their photochemical activity during rehydration. Even more pronounced differences became in agreement with the results from Table 17.1, showing that damage sustained during dehydration become particularly detrimental after full metabolic activity have set in after spinach leaves rehydration.

The most important result emerging from TL studies of *H. rhodopensis* leaves is that severe dehydration of resurrection plants affects mainly the number of PSII reaction centers, judging from significant decrease of B band amplitude (Figure 17.4, left panels) without any changes in the energetic state of the remaining operative centers. After rehydration of desiccated *H. rhodopensis* leaves, the number and the oscillation pattern of operating PSII centers were nearly completely



**FIGURE 17.4** TL curves of fully hydrated, dehydrated, and rehydrated *Haberlea rhodopensis* leaves after excitation by one saturating flash without DCMU, or in the presence of 20  $\mu$ M DCMU (A, C, E). TL B band oscillations as a function of flash number for corresponding state of dehydration (B, D, F). Amplitudes were normalized at the second flash. The fully hydrated leaves were dehydrated in the dark to 60% RWC (A, B), 40% RWC (C, D), 20% RWC (E, F) and rehydrated in moist filter paper for 24 h. Leaf disks with a diameter of 10 mm were used in the experiments.

restored (Figure 17.4, right panels). This process was very rapid and rehydration for only 2 h restored more than 80% of the initial B band amplitude.

The effect of desiccation and subsequent rehydration on the redox functioning of PSII donor and acceptor side redox components of *H. rhodopensis* leaves was also assessed by the changes in the main TL bands emitted at illumination with continuous white light during cooling the leaf disks from room temperature to  $-20^{\circ}\text{C}$ . Under these experimental conditions a complex glow curve with well-resolved TL bands at about  $0^{\circ}\text{C}$  and  $45^{\circ}\text{C}$ , corresponding to Q and B bands [52] was obtained. Representative TL curve pattern from the leaves of fully hydrated *H. rhodopensis* plants is shown in Figure 17.5A.

The traces in Figure 17.5B and C reveal that increasing dehydration resulted in clear changes in the overall intensity of TL signals and redistribution of the TL emission between the existing Q and B bands with practically unchanged peak temperatures. In desiccated leaves, the amplitude of the TL B

band ( $S_{2(3)}Q_B^-$ ) sharply decreased and mainly a charge recombination related to  $S_2Q_A^-$  (Q-peak) takes place (Figure 17.5C). After rehydration TL glow curve pattern resembles that of the control (fully hydrated) leaves (Figure 17.5D), which means electron transport between the primary and secondary electron acceptors was reversibly modified. Analogical changes in the amplitude and oscillation pattern of the main TL B and Q bands, obtained during flash illumination [53], suppose that some changes in the kinetic characteristics of  $S_2$  and  $S_3$  states of PSII donor side during desiccation cannot be excluded.

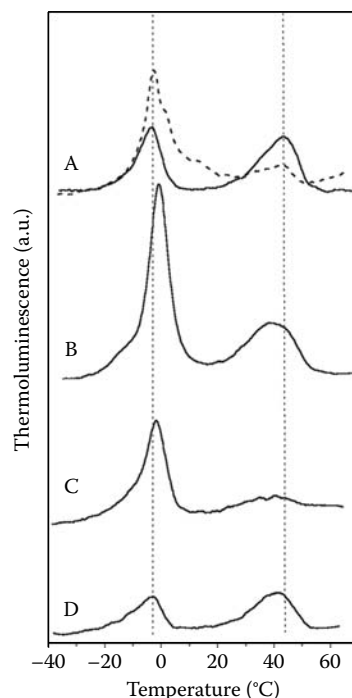
We suggested that the increased contribution of  $S_2Q_A^-$  charge recombination in dehydrated *H. rhodopensis* leaves served to protect  $Q_B$  site from over excitation. There are data that the increased population of  $Q_A^-$  enhances the probability for non-radiative energy dissipation and represents an effective mechanism of protection [48].

#### 17.4 PHOTOSYSTEM II REACTIONS IN CHLOROPLASTS MEMBRANES ISOLATED FROM FULLY HYDRATED AND DEHYDRATED *HABERLEA* *RHODOPENSIS* LEAVES

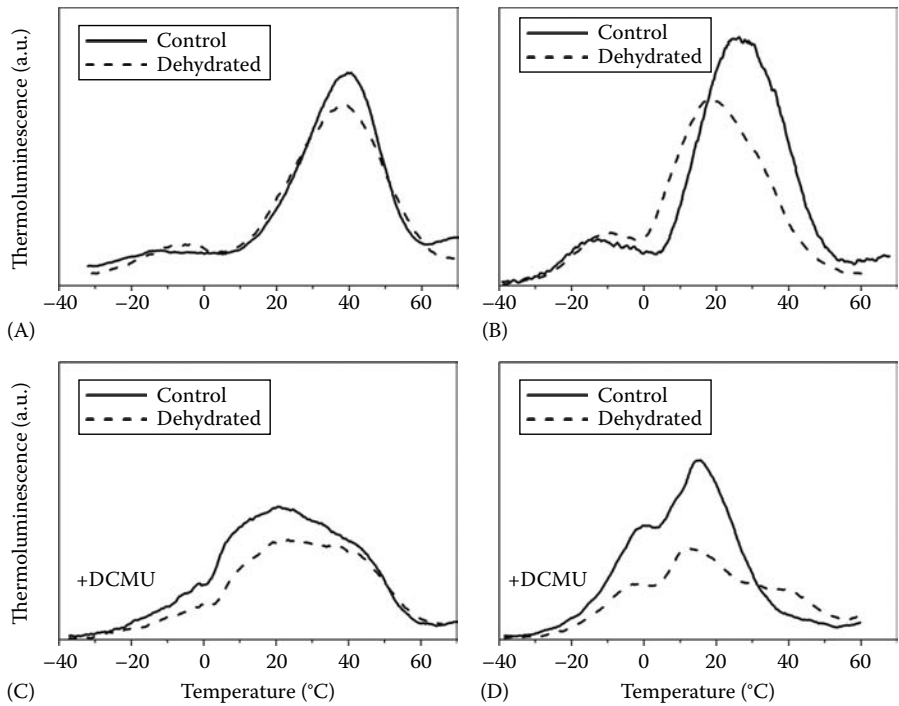
Answering the question whether the unique TL properties of the chlorophyll molecules of *H. rhodopensis* leaves were determined by some structural peculiarities required TL to be measured on isolated thylakoids. The obtained results show that the isolated photosynthesizing membranes from resurrection plant retain to a great extent the TL pattern of intact leaves thus indicating they were intrinsic features of PSII complex of *H. rhodopensis*.

The illumination of dark-adapted chloroplast suspensions isolated from fully hydrated *H. rhodopensis* leaves by continuous white light revealed a glow curve with a B band temperature maximum positioned at about 40°C (Figure 17.6A). The respective maximum in spinach chloroplast membranes appeared at much lower temperature of 26°C. The addition of DCMU to chloroplast suspension downshifted the B band emission maximum with formation of Q band at lower temperature (Figure 17.6C and D), but a residual B band like those in the leaves infiltrated with the electron-transport inhibitor was also observed, confirming the lower affinity of  $Q_B$  binding from PSII acceptor side in *H. rhodopensis* chloroplasts to DCMU.

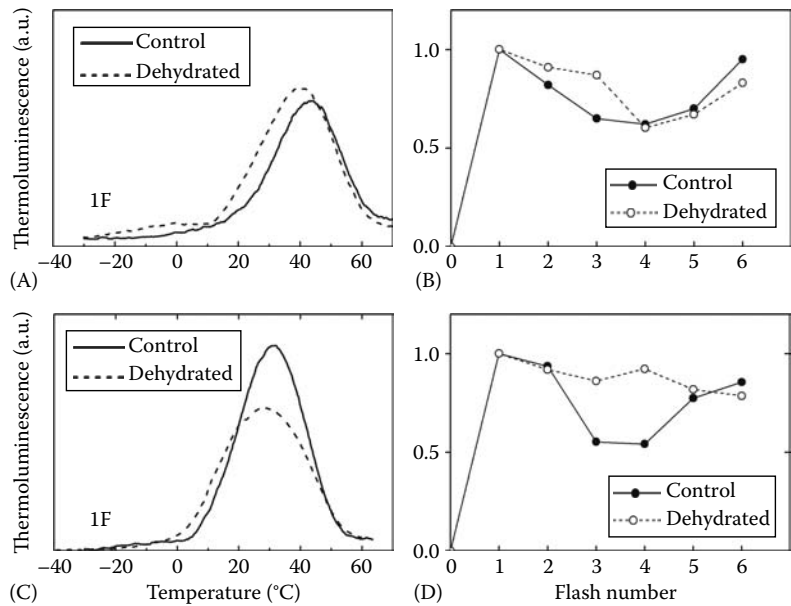
TL emission pattern of chloroplasts isolated from desiccated to 20% RWC *H. rhodopensis* leaves was identical to those isolated from fully hydrated plants (Figure 17.6A and C). The same maximal temperature position of B- and Q bands obtained in the both varieties of chloroplasts evidenced for a stable energetic state of recombination pairs, moreover, the reduction in overall TL intensity was found to be negligible. This is an indication that the chloroplasts isolated from desiccated leaves have been completely rehydrated when setting in resuspension medium and their functional activity was fully recovered in conformity with the preserved membrane integrity (Table 17.1). A significant downshift in temperature maximum and the decrease in the intensity of the respective TL bands in chloroplasts isolated from desiccated spinach leaves (Figure 17.6B and D) are a consequence of the membrane injuries occurred during severe stress.



**FIGURE 17.5** TL curves of *Haberlea rhodopensis* dark-adapted leaves after illumination by continuous white light of  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  from room temperature to  $-20^\circ\text{C}$  for 1 min. (A) Fully hydrated leaves 95% RWC and leaf disks infiltrated with  $20 \mu\text{M}$  DCMU (in dots). (B) Dehydrated leaves, 50% RWC. (C) Desiccated leaves, 10% RWC. (D) Rehydrated leaves for 24 h. Leaf disks with a diameter 10 mm were used in the experiments.



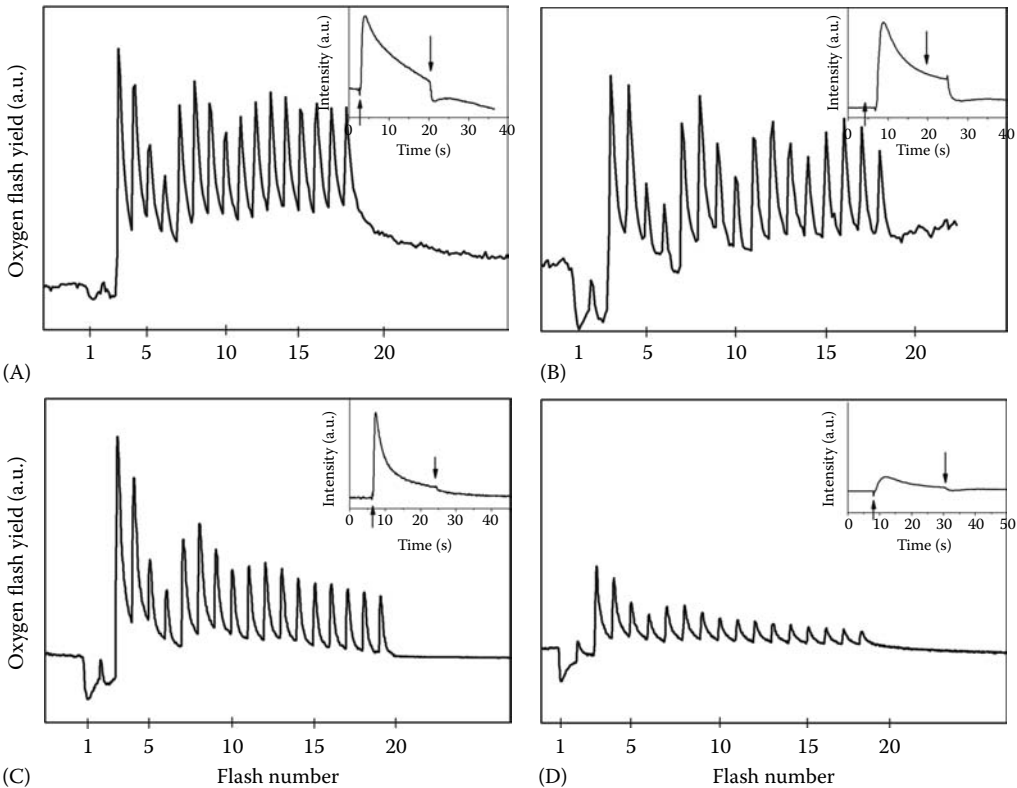
**FIGURE 17.6** TL emissions of *Haberlea rhodopensis* (A, C) and spinach (B, D) chloroplasts excited by continuous white light of  $150\mu\text{mol m}^{-2}\text{s}^{-1}$  at  $-20^\circ\text{C}$  for 1 min. The chloroplasts were isolated from fully hydrated leaves (solid lines) and leaves dehydrated to 20% RWC (dashed lines). Dark-adapted chloroplasts of  $1\text{ mg Chl mL}^{-1}$  were used in the experiments.



**FIGURE 17.7** TL B band of chloroplasts isolated from fully hydrated and dehydrated to 20% RWC leaves of *Haberlea rhodopensis* (A) and spinach (C) after excitation by one saturating flash. B band oscillations for *Haberlea rhodopensis* (B) and spinach (D) after excitation by flash sequences from 0 to 6. Amplitudes were normalized at the first flash. Dark-adapted chloroplasts of  $1\text{ mg Chl mL}^{-1}$  were used.

Changes in B band parameters (Figure 17.7A and C) and TL oscillation pattern (Figure 17.7B and D) of desiccated *H. rhodopensis* and spinach chloroplasts illuminated with saturated flashes compared to respective hydrated controls demonstrated once more that PSII reaction centers functioning was preserved in a great extent in resurrection plant.

Another reliable approach to study the properties of PSII complex in *H. rhodopensis* chloroplasts was to compare the kinetics of oxygen-evolving reactions with those of spinach membranes. The obtained results (Figure 17.8) demonstrated significant differences in the degree of inhibition of oxygen flash yields and the amplitude of initial oxygen burst between resurrection and mesophytic



**FIGURE 17.8** Oxygen flash yields sequences of chloroplasts isolated from fully hydrated (A) and dehydrated to 20% RWC (C) *Haberlea rhodopensis* leaves. Fully hydrated (B) and dehydrated to 20% RWC (D) spinach leaves. Insert: Oxygen evolution burst induced by 135 W m<sup>-2</sup> continuous white light. Dark-adapted chloroplasts of 0.3 mg Chl mL<sup>-1</sup> were used. The experiments were performed by a polarographic oxygen rate electrode with short (10 μs) saturating (4 J) flash sequences or continuous white light.

**TABLE 17.2**  
**Changes in the Kinetic Parameters of Oxygen-Evolving Reactions of Chloroplasts Isolated from Fully Hydrated and Desiccated *Haberlea rhodopensis* and Spinach Leaves, according to Kok's Model**

| Species         | RWC (%) | $S_0 + S_1$ (a.u.) | Misses ( $\alpha$ ) | Double Hits ( $\beta$ ) |
|-----------------|---------|--------------------|---------------------|-------------------------|
| <i>Haberlea</i> | 95      | 238.7 ± 6          | 0.151 ± 0.013       | 0.031 ± 0.004           |
| <i>Haberlea</i> | 20      | 213.1 ± 3          | 0.161 ± 0.008       | 0.027 ± 0.001           |
| Spinach         | 95      | 265.3 ± 11         | 0.137 ± 0.005       | 0.029 ± 0.002           |
| Spinach         | 20      | 54.2 ± 3           | 0.321 ± 0.008       | 0.020 ± 0.002           |

plants. The damping of oscillations of control chloroplasts and the chloroplasts, isolated from desiccated *H. rhodopensis* and spinach leaves was in accordance to the changes in the respective kinetic parameters, calculated by Kok's model [54]. As shown in Table 17.2, the desiccation of *H. rhodopensis* leaves do not change significantly the initial distribution of oxygen-evolving centers in  $S_0$  and  $S_1$  states and the values of misses ( $\alpha$ ) and double hits ( $\beta$ ). Alterations in the values of these parameters and in the shape of oxygen induction curve in desiccated spinach chloroplasts are indicative for membrane damage in the desiccation-intolerant plant.

## 17.5 CONCLUSION

The homoiochlorophyllous resurrection plant *Haberlea rhodopensis* Friv., demonstrated a deeper stabilization of PSII charge pairs, evidenced by an unusually high temperature maximum of the main TL B peak in the leaves and isolated thylakoid membranes. In addition, a part of these centers was less susceptible to DCMU, an inhibitor of electron transport. These features, as well as the strong reduction of the number of active PSII centers performing  $S_{2(3)}Q_B^-$  charge separation during desiccation without any changes in the energetics of the charge recombination in the rest operating centers were considered to indicate modifications of the redox properties of  $Q_B$ , related to desiccation tolerance of *H. rhodopensis*. It is reasonable to suggest that these modifications favor  $S_2Q_A^-$  charge recombination under desiccation. The increased population of  $Q_A^-$  enhances the probability for non-radiative energy dissipation and can represent an effective mechanism of protection during unfavorable environmental conditions.

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# 18 Carbon Metabolism and Plant Stress

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and Florencio E. Podestá*

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## 18.1 INTRODUCTION

Carbohydrates are the most abundant compounds in living organisms. However, their importance does not purely rely in abundance, as they play key roles in cell functionality. In plants, carbohydrates are utilized for the synthesis of various structural components, and they are used to transport carbon and energy between tissues (Iglesias and Podestá 2005, Smith 1999). The central role of carbohydrates in the energetic metabolism is highlighted in the glycolytic and the oxidative pentose-P pathways. However, these pathways have another important role, as they serve as source of intermediates for the synthesis of a large number of cellular components, such as lipids, nucleic acids, organic acids, and proteins. Also, carbohydrates are fundamental components of structural molecules like cellulose, the most abundant biomolecule. In addition, sugars can be combined with other compounds, like lipids or proteins, to produce glycolipids and glycoproteins, which play key roles related with cell structure and function (Iglesias and Podestá 2005, Smith 1999). On the other hand, carbohydrates are the major reserve and mobilization constituents in plants, where they can be found as starch, sucrose, fructans, and polyols (Iglesias and Podestá 2005, Loescher and Everard 2004, Smith 1999).

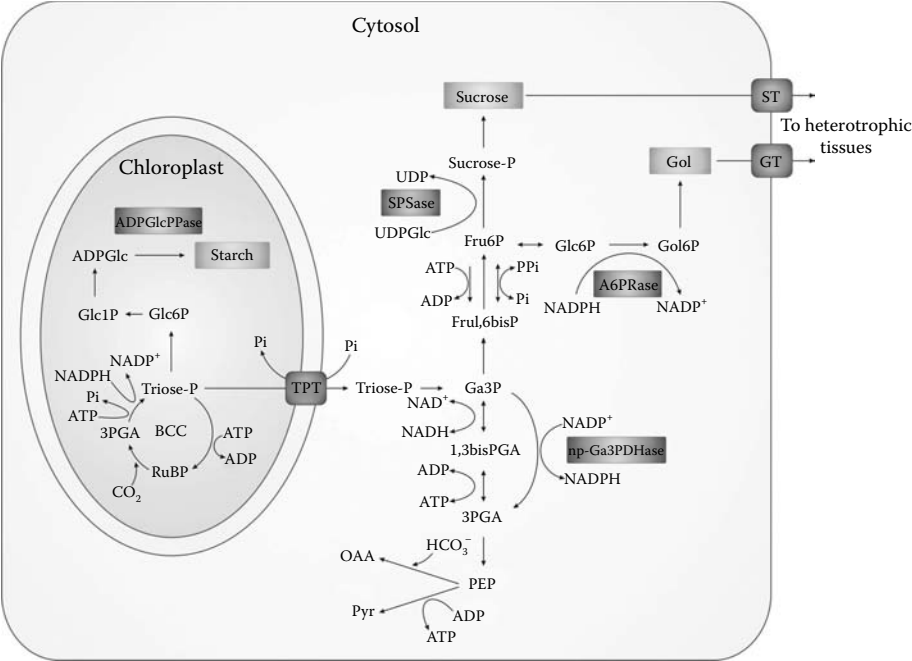
The sessile lifestyle of plants imposes severe challenges to their ability to survive in a changing environment. Plants are affected by a variety of stresses including, but not limited to, water shortage, nutrients deficiency, presence of toxic compounds (natural or arising from contamination), and temperature extremes. A series of mechanisms have been developed by plants that allow the adjustment of the metabolic machinery to cope with unfavorable conditions, which in fact may be the prevailing situation in the wild. Carbon metabolism contributes in various ways with the adaptation to a stressful situation. Relocation of carbon skeletons for the synthesis of specific stress-related metabolites, excretion of organic acids to confront the presence of toxic metals, or to minimize Pi deficiency or alterations in the main course of metabolic pathways through alternative routes are some of the responses observed. Metabolic adaptations include the transient adjustment of constitutive processes or the induction of latent ones to accommodate to a new condition by fine-tuning metabolism. More extensive changes can even include the adaptation of the whole photosynthetic mechanism of CO<sub>2</sub> assimilation as a result of a long-term challenge.

This chapter deals with the main adjustments of plant primary carbon metabolism to the most common forms of abiotic stress, which endow these organisms with the capacity to survive in a world of permanently changing situations. The outstanding role of carbohydrates for carbon and energy metabolism forces to highlight their involvement as key components of cellular processes. We analyze the different fates of carbohydrates produced by photosynthetic assimilation in plants. The analysis emphasizes on how metabolic routes mainly operating in the cytosol are critical to direct carbohydrate fluxes to produce energy, reduce power, and transport or reserve molecules. The overall picture seeks to understand relationship between carbohydrates and plant physiology, and the capacity to cope with different stress conditions.

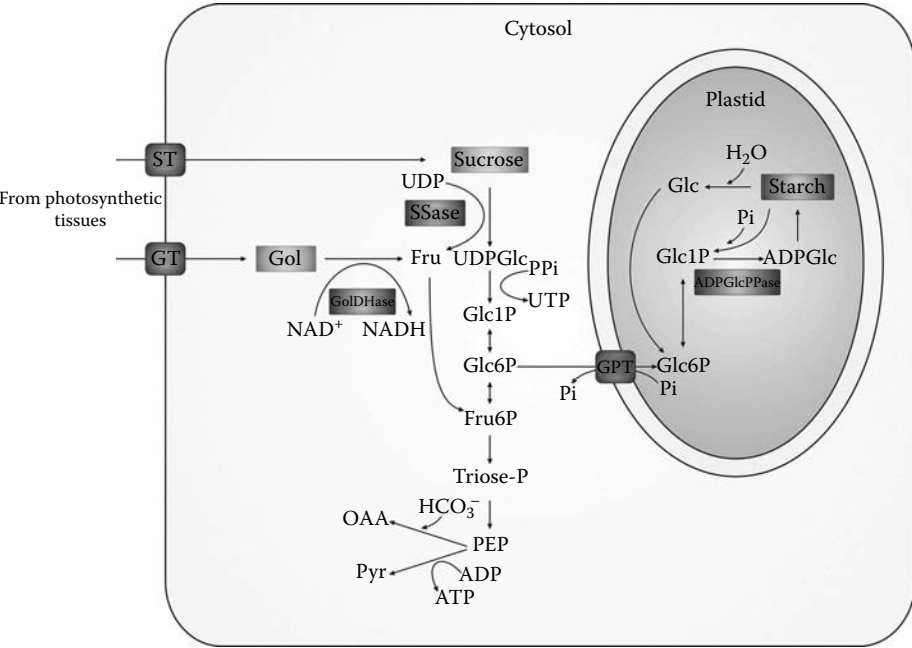
## 18.2 CARBON PARTITIONING IN PLANTS

Oxygenic photosynthesis is a process where the energy from light is utilized for carbohydrate production, thus playing a key role on Earth, because most organisms depend directly or indirectly on it to obtain energy. In the global photosynthetic process, the occurrence of two phases can be differentiated: (a) a light phase, where the electromagnetic sun energy is converted into chemical energy (ATP) and reducing power (NADPH) and (b) a synthetic phase, comprising the use of ATP and NADPH to fix atmospheric CO<sub>2</sub> to primarily generate carbohydrates. As shown in Figure 18.1, carbon fixation occurs in the chloroplast of the photosynthetic plant cell mainly through the Benson–Calvin cycle (BCC), utilizing ATP and NADPH produced in the light phase. This renders triose-P and hexose-P that can be used within chloroplasts for the production of starch. Alternatively, photosynthates (mainly in the form of triose-P) can be exported to the cytosol to be used in glycolytic routes as well as to synthesize sucrose or polyols, the major compounds used for carbon translocation from source to sink tissues. In this way, photoassimilates are subjected to two levels of partitioning. At the intracellular level, they are partitioned between the chloroplast and the cytosol, being triose-P the key intermediaries interexchanged with Pi through a specific translocator of the plastid envelope (TPT in Figure 18.1). In the cytosol, carbohydrates are derived to different routes, including glycolysis and synthesis of soluble sugars that are accumulated in this subcellular compartment and also utilized for transport to other parts of the plant through the phloem. In most plants, the major soluble sugar synthesized is sucrose, although many species also produce sugar-alcohols (e.g., glucitol, abbreviated Gol, see Figure 18.1) as a main metabolite. The second level of partitioning takes place as carbohydrates are distributed to other tissues having heterotrophic characteristics, being sucrose (or a sugar-alcohol in certain species) the main metabolite transported between different tissues. Once in the non-photosynthetic cell, sucrose (or the sugar-alcohol) is derived to different metabolic routes with the establishment, once again, of a partitioning of photoassimilates between the cytosol and the plastid (Figure 18.2).

As described above, carbohydrates are partitioned between a relatively stationary (starch) and a mobile (sucrose, polyols) form as major products of photosynthesis. It is important to understand the



**FIGURE 18.1** Schematic representation of carbon metabolism in plant photosynthetic cells. Key enzymes for carbon partitioning, the major photosynthetic products (starch, sucrose, and glucitol), and transporters (TPT, triose-P translocator; ST, sucrose transporter; GT, glucitol transporter) are highlighted. BCC is the abbreviation of Benson–Calvin cycle.



**FIGURE 18.2** Schematic representation of carbon metabolism in plant heterotrophic cells. Enzymes involved in sucrose, starch, and glucitol metabolism, photosynthates translocated from leaves (sucrose and glucitol), starch (the major storage compound), and the glucose-P translocator (GPT) are highlighted.

processes of synthesis, partition (within one cell and between source and sink tissues), and storage of carbohydrates because these molecules are crucial for plant productivity. The control of these processes is affected by different factors, at systemic or cellular levels, which may modify carbon and energy demands of different tissues (Iglesias and Podestá 2005). Starch biosynthesis takes place in the chloroplast (Figure 18.1) and involves three enzymatic steps, sequentially catalyzed by (see Equations 18.1 through 18.3, respectively, in Figure 18.3) ADP-glucose pyrophosphorylase (ADPGlcPPase, EC 2.7.7.27), starch synthase (EC 2.4.1.21), and branching enzyme (EC 2.4.1.18) (Ballicora et al. 2003, 2004). This polyglucan is the major storage compound in higher plants, and in photosynthetic and heterotrophic tissues. It has been established that starch constitutes a transitory storage glucan in source tissues and its level varies during the photoperiod; while the long-term storage occurs in the plastids of heterotrophic cells in non-photosynthetic tissues, such as fruits, roots, and tubers (Figure 18.2) (Iglesias and Podestá 2005).

|                                                                                                                |         |
|----------------------------------------------------------------------------------------------------------------|---------|
| $\text{Glc1P} + \text{ATP} \rightleftharpoons \text{ADPGlc} + \text{PPi}$                                      | (18.1)  |
| $\text{ADPGlc} + (\alpha\text{-1,4-glucan})_n \rightarrow \text{ADP} + (\alpha\text{-1,4-glucan})_{n+1}$       | (18.2)  |
| Linear $\alpha\text{-1,4-glucan chain} \rightarrow \alpha\text{-1,6-branched } \alpha\text{-1,4-glucan chain}$ | (18.3)  |
| $\text{Fru6P} \rightleftharpoons \text{Glc6P}$                                                                 | (18.4)  |
| $\text{Glc6P} \rightleftharpoons \text{Glc1P}$                                                                 | (18.5)  |
| $\text{Fru6P} + \text{ATP} \rightarrow \text{Fru1,6bisP} + \text{ADP}$                                         | (18.6)  |
| $\text{Fru6P} + \text{PPi} \rightleftharpoons \text{Fru1,6bisP} + \text{Pi}$                                   | (18.7)  |
| $\text{Ga3P} + \text{NAD}^+ + \text{Pi} \rightleftharpoons \text{1,3PGA} + \text{NADH} + \text{H}^+$           | (18.8)  |
| $\text{Ga3P} + \text{NADP}^+ + \text{H}_2\text{O} \rightarrow \text{3PGA} + \text{NADPH} + 2 \text{H}^+$       | (18.9)  |
| $\text{PEP} + \text{HCO}_3^- \rightarrow \text{OAA} + \text{Pi}$                                               | (18.10) |
| $\text{PEP} + \text{ADP} \rightarrow \text{Pyr} + \text{ATP}$                                                  | (18.11) |
| $\text{PEP} + \text{ADP} + \text{HCO}_3^- \rightleftharpoons \text{OAA} + \text{ATP}$                          | (18.12) |
| $\text{UDPGlc} + \text{Fru6P} \rightleftharpoons \text{Sucrose-P} + \text{UDP}$                                | (18.13) |
| $\text{Sucrose-P} + \text{H}_2\text{O} \rightarrow \text{Sucrose} + \text{Pi}$                                 | (18.14) |
| $\text{Sucrose} + \text{H}_2\text{O} \rightarrow \text{Glc} + \text{Fru}$                                      | (18.15) |
| $\text{Sucrose} + \text{UDP} \rightleftharpoons \text{UDP-Glc} + \text{Fru}$                                   | (18.16) |
| $\text{Glc6P} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Gol6P} + \text{NADP}^+$                     | (18.17) |
| $\text{Gol6P} + \text{H}_2\text{O} \rightarrow \text{Gol} + \text{Pi}$                                         | (18.18) |
| $\text{Gol} + \text{NAD}^+ \rightleftharpoons \text{Fru} + \text{NADH} + \text{H}^+$                           | (18.19) |

**FIGURE 18.3** Key reactions catalyzed by enzymes involved in carbohydrate metabolism and partitioning in plants.

### 18.3 CARBON METABOLISM IN THE CYTOSOL AND PLANT STRESS

Many of the pathways of carbohydrate metabolism take place in the cytosol, where they are connected to other metabolic routes. In this compartment, there is a metabolic node constituted by fructose-6-P (Fru6P), glucose-6-P (Glc6P), and glucose-1-P (Glc1P). The conversion between Fru6P and Glc6P (see [Figure 18.3](#), Equation 18.4) is catalyzed by a hexose-P isomerase (EC 5.3.1.9) while the reaction that converts Glc6P into Glc1P (Equation 18.5 in [Figure 18.3](#)) is catalyzed by a phosphoglucomutase (EC 5.4.2.2). In plants, the hexose-P pool can be utilized to produce many other metabolites, thus directing carbon skeletons to different metabolic fluxes (Smith 1999). The main fates for cytosolic hexose-P include glycolysis and synthesis of soluble sugars as sucrose and polyols. These central metabolic routes produce intermediate metabolites or final products that play critical roles in plants under physiological as well as stress conditions.

#### 18.3.1 ORGANIZATION OF PLANT GLYCOLYSIS

Glycolysis is a ubiquitous metabolic pathway that converts glucose (Glc) into pyruvate (Pyr) in most organisms, producing 2 ATPs as a result (Givan 1999, Plaxton 1996, Podestá 2004). Glycolysis is just the first step of respiration, the fundamental process of intermediary metabolism. In plants, glycolysis is unique in that it possesses a series of differential features and its function goes beyond the provision of ATP and Pyr for mitochondrial respiration (Plaxton and Podestá 2006). One of these characteristics is the ability to use PPi instead of ATP as phosphoryl donor (Plaxton and Podestá 2006, Stitt 1998). Also, ancillary enzymes can be found that expand the classic 10 reactions pathway and lend a great degree of flexibility to metabolism (Plaxton 1996). Most important, the final product of cytosolic plant glycolysis is not necessarily Pyr. Instead, the cytosolic pool of phosphoenolpyruvate (PEP) represents a metabolic branch point from which carbon skeletons may follow different fates (Plaxton 1996, Plaxton and Podestá 2006). In addition, Glc is not the obligated starting metabolite for glycolysis: the photosynthetically generated hexose-P or triose-P pools can both contribute with carbon skeletons in the route toward PEP or Pyr (Dennis and Blakeley 2000). Finally, it must be mentioned that plants possess a second set of glycolytic enzymes in the plastid, with its own distinctive characteristics. All of these features can be efficiently exploited by plants to face different types of stress situations.

Starting from the top, down along the conventional glycolytic pathway, the first distinguishing characteristic of the plant cell cytosolic glycolytic metabolism arises at the level of the conversion of Fru6P to Fru1,6bisP. As in most other organisms, it is a key regulatory step, but the phosphorylation reaction can be achieved by two different enzymes (see, respectively, Equations 18.6 and 18.7 in [Figure 18.3](#)): a classical but Fru2,6bisP-insensitive ATP-dependent phosphofructokinase (ATP-PFKase, EC 2.7.1.11) or a PPi-dependent enzyme that uses PPi as phosphoryl donor (PPi-PFKase, EC 2.7.1.90) and is potently activated by Fru2,6bisP (Carnal and Black 1979, Dennis and Blakeley 2000, Givan 1999, Iglesias and Podestá 2005, Plaxton and Podestá 2006) ([Figure 18.1](#)). Plant PPi-PFKase is widespread in different plant species and tissues and its subunit composition and activity respond to environmental and developmental cues (Nielsen 1995, Plaxton and Podestá 2006, Podestá and Plaxton 1994a, Theodorou and Plaxton 1996, Trípodí and Podestá 1997). Current knowledge indicates that it most probably works in the glycolytic direction, despite the fact that it catalyzes a reversible reaction, controlling the balance between the triose-P and hexose-P pools and carbon partitioning among starch and sucrose (Hajirezaei et al. 1994, Plaxton 1996, Plaxton and Podestá 2006, Podestá and Plaxton 2003).

Following conversion of Fru1,6bisP into triose-P, the first energy-conserving reaction takes place with the generation of ATP by the joint action of glyceraldehyde 3-P dehydrogenase (Ga3PDHase, EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3). The first reaction (Equation 18.8 in [Figure 18.3](#)), the only oxidation that takes place during glycolysis, can be circumvented by a NADP-dependent, non-phosphorylating glyceraldehyde 3-P dehydrogenase (np-Ga3PDHase, EC 1.2.1.9)

that yields 3PGA and uses NADP rather than NAD as acceptor of the reducing equivalents (Bustos and Iglesias 2002, Iglesias et al. 2002) (see [Figure 18.3](#), Equation 18.9). The reaction is irreversible in this case, and leads to a product that cannot be used to generate ATP. The contribution of this enzyme thus leads to a nil ATP yield. However, the importance of np-Ga3PDHase in plant cytosolic metabolism is beginning to be acknowledged, having been implicated in fruit development and energetic metabolism. A recent work (Bustos et al. 2008) points out that the np-enzyme is implicated in the response to oxidative stress in wheat leaves. Also, plants deficient in this enzyme were found to exhibit a reduced glycolytic capacity and increased levels of oxidative stress (Rius et al. 2006).

From then on, two reactions lead to the production of PEP. As stated above, PEP occupies a central role in plant carbohydrate metabolism as a regulatory molecule, but it is also significant as a branchpoint metabolite. PEP is the substrate for a carboxylation reaction (see [Figure 18.3](#), Equation 18.10), mediated by PEP carboxylase (EC 4.1.1.31), a regulatory enzyme that produces oxaloacetate (OAA) and Pi (Chollet et al. 1996, Iglesias et al. 1997) ([Figure 18.1](#)). PEP carboxylase exists in various isoforms and different levels in plant tissues. It plays a major role in anapleurosis replenishing intermediates of the tricarboxylic acid pathway, and thus links cytosolic carbon metabolism with respiration and N assimilation. In  $C_4$  and Crassulacean acid metabolism plants the enzyme is responsible for the primary  $CO_2$  fixation, starting the  $CO_2$  concentrating mechanism. PEP carboxylase is subjected to tight regulation by metabolites and by reversible phosphorylation on a serine residue (Bakrim et al. 2001, Chollet et al. 1996, Trípodí and Plaxton 2005). Malate is a strong inhibitor of PEP carboxylase, but its effect is dependent on pH and, most important, on the phosphorylation status of the enzyme. The phosphorylated form of PEP carboxylase is usually more active and less sensitive to malate, specially at pH values on the acidic side of the optimum, which is around pH 8.0 (Baur et al. 1992, Chollet et al. 1996, Hartwell et al. 1999, Moraes and Plaxton 2000, Trípodí and Plaxton 2005). Glc6P is an important allosteric effector, promoting activation of the enzyme and reducing malate sensitivity (Chollet et al. 1996, Plaxton 1996). The enzyme from  $C_3$  plants (i.e., banana fruit and castor bean germinating endosperm) is very sensitive to inhibition by aspartate and glutamate, highlighting the link between N and C metabolism mediated by PEP carboxylase (Law and Plaxton 1995, Trípodí and Plaxton 2005). Phosphorylation alleviates the effect of aspartate (Gregory et al. 2009, Law and Plaxton 1995, Trípodí and Plaxton 2005). The characterization of PEP carboxylase kinase has shown that its ability to phosphorylate the target enzyme is impaired in the presence of malate, while it performs better in the presence of PEP (Murmu and Plaxton 2007).

PEP is also the substrate for the usual end of glycolysis in most organisms, the reaction catalyzed by Pyr kinase (PyrKase, EC 2.7.1.40) that produces Pyr and ATP (see [Figure 18.3](#), Equation 18.11) and constitutes the second energy-conserving reaction. PyrKase has been studied in many plant tissues where its regulatory role of the whole glycolytic process has been recognized (Hu and Plaxton 1996, Lin et al. 1989, Moraes and Plaxton 2000, Plaxton 1989, Podestá and Plaxton 1991). In fact, the activity of this enzyme is instrumental in controlling the flux of the upper part of glycolysis by regulating the levels of PEP (Givan 1999, Plaxton 1996), as it will be further analyzed below. PyrKase regulation is organ specific in plants. The cytosolic PyrKase from germinating castor seed endosperm or cotyledons shows a pH-dependent response to several metabolite inhibitors (Podestá and Plaxton 1991, Podestá and Plaxton 1994b). A concerted decrease in pH and inhibitor concentrations, as could be caused by anoxia, will cause PyrKase activity to rise. Glutamate is an important inhibitor of the enzyme from several sources (Hu and Plaxton 1996, Lin et al. 1989, Plaxton 1996, Plaxton and Podestá 2006, Podestá and Plaxton 1994b), while aspartate activates PyrKase and relieves from the inhibitory effect of the former. The reciprocal effects of aspartate on PyrKase and PEP carboxylase provide an effective mechanism to balance both activities during active N assimilation. Thus, aspartate accumulation reduces the flux through PEP carboxylase while enhancing PyrKase activity in leaves, ripening banana fruit and castor bean endosperm (Plaxton and Podestá 2006, Smith et al. 2000, Turner et al. 2005, Turner and Plaxton 2000).



A third enzyme also makes use of PEP. PEP carboxykinase (PEPCKase, EC 4.1.1.49) catalyzes the reversible carboxylation of PEP, using ADP as substrate as well, to yield OAA and ATP (Chen et al. 2004, Daley et al. 1977, Leegood and Walker 2003, Rylott et al. 2003, Walker and Leegood 1996, Wingler et al. 1999) (Figure 18.3, Equation 18.12). Although generally implicated in gluconeogenesis, the activity of PEPCKase is also crucial to determine the concentration of PEP. It has been shown that the enzyme is subjected to phosphorylation in many but not all plants (Leegood and Walker 2003). The study of PEPCKase has been hindered by the extreme sensitivity of the enzyme to proteolysis upon extraction (Martín et al. 2007). The truncated, but otherwise active enzyme exhibits few, if any, regulatory properties. The phosphorylation site lies within the excised peptide, which strongly suggest that whatever changes in the regulation of PEPCKase are brought about by phosphorylation are not evident on the proteolyzed enzyme (Leegood and Walker 2003). So far, studies with a preparation containing only the phospho or dephospho forms of PEPCKase are lacking, but the use of special reaction media designed to measure preferentially one form demonstrated that phosphorylation lowers PEPCKase activity (Leegood and Walker 2003). A strict coordination in the phosphorylation status of PEP carboxylase and PEPCKase is necessary as the two enzymes acting at the same time can give rise to a potential futile cycle.

Levels of PEP thus depend on the coordination of the activities of at least three regulatory enzymes. The importance of this can be appreciated after examination of the role of PEP as a regulatory metabolite. In plants, the conversion of Fru6P to Fru1,6bisP does not represent the primary control point of glycolysis, in part due to the lack of activation of ATP-PFKase by Fru2,6bisP. PPI-PFKase, instead, is strongly activated by Fru2,6bisP (Kruger and Dennis 1987, Theodorou and Plaxton 1994, Trípodi and Podestá 1997). To date, all plant cytosolic ATP-PFKases examined are inhibited by PEP (Givan 1999, Lee and Copeland 1996), and this metabolite also is an inhibitor of the synthesis of Fru2,6bisP (Plaxton and Podestá 2006). Levels of PEP, thus, control the flux through the upper part of glycolysis, reinforcing the role of PEP carboxylase and PyrKase as pacemaker enzymes of the whole pathway. This does not mean that control is completely delegated on these two enzymes; rather that the primary control depends on them. ATP-PFKase and PPI-PFKase are still relevant for the fine-tuning of carbon flow in the cytosol, with activities that depend on the levels of PEP but also on Pi concentration, as Pi activates ATP-PFKase while it is a powerful inhibitor of the glycolytic reaction of PPI-PFKase (Plaxton and Podestá 2006, Podestá 2004). In any case, the study of the contribution of ATP-PFKase, PPI-PFKase, PEP carboxylase, and PyrKase by the use of transgenic lines deficient in or overexpressing these enzymes is complicated by the great degree of metabolic flexibility that plants show. In many cases, successful creation of a transgenic plant does not result in an evident phenotype, or it displays it only under a particular condition (Grodzinski et al. 1999, Hajirezaei et al. 1994, Stitt and Sonnewald 1995).

### 18.3.2 METABOLISM OF SUCROSE AND POLYOLS

In leaves, during the light period, sucrose is produced from triose-P translocated from the chloroplast through the TPT in exchange for Pi (Figure 18.1). In the dark, starch mobilization allows the constant production of sucrose and the uninterrupted flow of carbon to heterotrophic tissues (Winter and Huber 2000). The reactions that derive to sucrose synthesis include the action of sucrose-P synthase (SPSase, EC 2.4.1.14) and sucrose-P phosphatase (EC 3.1.3.24) (see Figure 18.1, and Equations 18.13 and 18.14 in Figure 18.3). Sucrose is then transported to sink tissues where its degradation can be catalyzed by two different classes of enzymes. Invertases (EC 3.2.1.26, Equation 18.15 in Figure 18.3) catalyze the irreversible hydrolysis of sucrose to Glc and Fru. On the other hand, a reversible cleavage of sucrose occurs by the action of sucrose synthase (SSase, EC 2.4.1.13, Equation 18.16 in Figure 18.3; see also Figure 18.2) (Iglesias and Podestá 2005, Winter and Huber 2000). The latter reaction conserves the energy of the glycosidic bond in UDP-glucose (UDPGlc), which can be used for cellulose synthesis or can enter glycolysis. In heterotrophic tissues, Glc6P or Glc1P are the preferred molecules carried



through a specific translocator (GPT, see [Figure 18.2](#)) inside plastids, where it can be utilized for starch synthesis (Winter and Huber 2000).

In addition to sucrose and starch, certain plants synthesize Gol (also known as sorbitol) or mannitol as important photosynthetic products (Loescher and Everard 2004). In these organisms, the analysis of [ $^{14}\text{C}$ ]CO<sub>2</sub> assimilation reveals the presence of two major soluble compounds: the polyol and sucrose. In apple leaves, about 70% of the newly photosynthetically fixed carbon was found as Gol and sucrose (Grant and Rees 1981). In celery, almost 80% of the label in mature leaves was recovered as mannitol and sucrose, with similar quantities (on a molar basis) of each one (Loescher et al. 1992). Similar results were obtained in apricot, where Gol was found to be the compound with the highest label 30 min after the pulse (Bielecki and Redgwell 1977). Gol is a major photosynthetic product in many important fruit-bearing tree species of the Rosaceae family, such as apple, peach, pear, and loquat. In general, a few studies deal with sugar-alcohols metabolism, in particular if compared with the abundant bibliography concerning starch (Ballicora et al. 2003, 2004) and sucrose (Koch 2004, Winter and Huber 2000) pathways. Because in many plants sugar-alcohols are main photosynthetic products, their metabolism is expected to be tightly regulated, probably at different levels. It is worth noting that sugar-alcohols could have important roles in plant tolerance to certain types of abiotic stresses (Loescher and Everard 2004).

Gol is synthesized in mature leaves through the conversion of Glc6P into Gol6P by the action of a NADPH-dependent aldose-6-phosphate reductase (A6PRase, EC 1.1.1.200; see Equation 18.17 in [Figure 18.3](#)) (Figuerola and Iglesias 2010, Hirai 1981, Kanayama and Yamaki 1993, Zhou et al. 2003b) and the subsequent hydrolysis of the phosphate group catalyzed by a specific Gol6P phosphatase (EC 3.1.3.50, Equation 18.18 in [Figure 18.3](#)) (Zhou et al. 2003a). These enzymes can be found in the cytosol in soluble forms, without any association with vacuoles, chloroplasts, mitochondria, peroxysomes, or membranes (Loescher and Everard 2004). The NADPH necessary for synthesizing the sugar-alcohol may be derived from the reaction catalyzed by np-Ga3PDHase (see [Figure 18.3](#), Equation 18.9), which has been found in celery leaves at levels capable of sustaining relevant production of mannitol by this plant (Gao and Loescher 2000, Rumpho et al. 1983). The sugar-alcohol produced in leaves can be transported to sink or developing tissues, such as fruits or immature leaves, where it can be converted to Fru by the action of a NAD-dependent Gol dehydrogenase (GolDHase, EC 1.1.1.14, Equation 18.19 in [Figure 18.3](#)) (Ohta et al. 2005, Oura et al. 2000, Yamaguchi et al. 1994).

## 18.4 PLANTS CARBOHYDRATE METABOLISM AND STRESS

### 18.4.1 RESPONSES OF GLYCOLYTIC CARBON METABOLISM ENZYMES TO STRESS

#### 18.4.1.1 Hexose-P Metabolism

Mertens et al. (1990) found that PPI-PFKase (but, not ATP-PFKase) is induced upon anoxia in rice seedlings. Fru2,6bisP also increases during anoxia in rice seedlings, helping to boost PPI-PFKase activity in a more acidic environment that could curtail this enzyme's activity (Mertens 1990). In this tissue, sucrose degradation proceeds mainly through the uridylate requiring the sucrose synthase/nucleoside diphosphate kinase system, lowering the dependence on adenylates that usually have lower levels upon this condition (Ricard et al. 1991). Pi starvation increases PPI-PFKase levels in *Brassica nigra* and *Brassica napus* (Theodorou and Plaxton 1994, 1996). *B. nigra* seedlings respond to Pi starvation by increasing the extractable activity of PPI-PFKase, the ratio PPI-PFKase:ATP-PFKase and by an increase in the amount of the  $\alpha$  relative to the  $\beta$  subunit (Theodorou and Plaxton 1994). The same response in the  $\alpha$ : $\beta$  ratio has been observed in black mustard suspension cells under Pi deficit (Theodorou et al. 1992). In both cases, the sensitivity to Fru2,6bisP is increased. Thus, the response to Pi stress at the hexose-P pool level is clearly to circumvent the adenylate-dependent step catalyzed by ATP-PFKase. This is a result of the acquired ability of plants to use PPI as a phosphoryl donor, efficiently using the energy available in the anhydride bond

(Stitt 1998). Assuming that PPi is a by-product of anabolism; no ATP is needed for the conversion of sucrose to hexose-P via the SSase pathway in heterotrophic tissues, whereas 2 ATPs are needed for the invertase pathway. Remarkably, although PPi can be hardly conceived as nothing more than a transient by-product of several cell reactions, which is readily hydrolyzed in animal cells, PPi levels in the cytosol of plant cells remain stable through a variety of conditions, including, but not limited to, severe Pi-deficiency or anoxia, which is consistent with the importance of PPi-dependent enzymes during stress (Plaxton and Podestá 2006, Stitt 1998). The importance of PPi in plant cell economy has been demonstrated by the creation of transgenic plants expressing a bacterial pyrophosphatase in the cytosol. These plants showed a threefold decreased PPi concentration and also severely impaired growth (Jellito et al. 1992).

The link between Pi nutritional status and the regulation of ATP-PFKase and PPi-PFKase is also evident from the fact that ATP-PFKase is regulated by the Pi:PEP ratio (with Pi being an activator and PEP an inhibitor); while Pi is a potent inhibitor of PPi-PFKase in the glycolytic direction (Dennis and Blakeley 2000). This suggests that under Pi deficiency, the latter would be the predominant activity, thus allowing an adenylate-independent glycolytic pathway. Pi-PFKase has been implicated in the response to cold stress as well. An examination of the subunit composition of PPi-PFKase in orange fruits showed a displacement of the  $\alpha:\beta$  subunit ratio from 1.66 to 1 upon exposure to frost and almost a doubling of its activity, all this complemented by an increased sensitivity to Fru2,6bisP (Falcone Ferreyra et al. 2006). Cold stress leads to an initial fall in sucrose synthesis, accumulation of phosphorylated metabolites, and consequent Pi-limitation of photosynthesis (Stitt and Hurry 2002). Plants respond to the challenge by increasing sucrose synthesis. Within minutes of initiation the cold stress in *Arabidopsis thaliana*, SPSase is posttranslationally activated by phosphorylation (Stitt and Hurry 2002). On the longer term, SPSase and cytosolic Fru1,6bisP phosphatase expression are boosted, releasing the Pi necessary for sustained photosynthesis at the expense of phosphorylated metabolites (Stitt and Hurry 2002). The response also includes movement of Pi from the vacuole to the cytosol, to allow the replenishment of phosphorylated metabolites without depleting free Pi (Hurry et al. 2000). Thus, as expressed by Stitt and Hurry (2002): “changes in Pi modulate and may even act as a signal in the regulation of photosynthetic/metabolic acclimation to low temperatures, and lead to major changes in the ability of the different genotypes to develop frost tolerance.”

#### 18.4.1.2 PEP Metabolism

Levels of PEP carboxylase have been reported to vary in response to different abiotic stresses. Recently, a study in frost-damaged orange fruits showed increased levels of PEP carboxylase in stressed fruit, which correlate with a lower sensitivity to its natural feedback inhibitor malate (Falcone Ferreyra et al. 2006). Since the fermentative pathway is higher in this tissue, it has been proposed that PEP carboxylase could act, in combination with malate dehydrogenase, as an ancillary fermentative enzyme, helping in the provision of ATP when aerobic respiration is affected (Falcone Ferreyra et al. 2006). Vu et al. (1995) reported that cold-hardy citrus varieties responded with an increase in extractable PEP carboxylase activity upon acclimation, whereas a sensitive cultivar showed a decrease in foliar PEP carboxylase levels. Similar trends have been reported in other cultivars exposed to cold (Vu et al. 1995), underscoring an as yet little studied role of PEP carboxylase in plant primary metabolism. Abiotic stresses that affect water balance, including cold stress and anoxia, also provoke an induction of PEP carboxylase. Induction was root-specific, except for the cold treatment, which also induced the enzyme in shoots (González et al. 2003). In general, induction of PEP carboxylase has been linked to an increased need for synthesis of organic acids such as malate, in response to cytoplasmic alkalization among other challenges.

PEP carboxylase activity is also responsive to Pi levels. Several studies showed an increase in PEP carboxylase content upon nutritional deprivation of Pi. One of the first papers describing the bypass of adenylate-using enzymes upon Pi deprivation in *B. nigra* suspension cells reported that PEP carboxylase, np-Ga3PDHase, PPi-PFKase, and PEP phosphatase showed important increases

in activity at the expense of the ATP-PFKase, PyrKase, and Ga3PDHase (Duff et al. 1989). *B. napus* efficiently uses rock Pi by excreting organic acids to the environment with the concurrent solubilization of the former. Pi deprivation in *B. napus* and the related hedge mustard caused an increase in PEP carboxylase levels and, probably as a result of this, also in malate exudation (Hoffland et al. 1992). Moraes and Plaxton (2000) reported the purification and properties of the Pi-deficient *B. nigra* PEP carboxylase. Pi starvation increased PEP carboxylase by 2.5-fold, while refeeding caused an immediate return to near control levels. The study of the purified enzyme showed that the increased activity under Pi shortage cannot be attributed to an increased phosphorylation state of the enzyme that would make it less susceptible to malate inhibition. It is possible that, at least in these cells, metabolite effects override the importance of phosphorylation in PEP carboxylase control (Moraes and Plaxton 2000). Several reports (Andaluz et al. 2009, Thimm et al. 2001) highlight the induction of PEP carboxylase in roots under iron deficiency. The induction of PEP carboxylase accompanies the apoplastic acidification caused by iron starvation (Thimm et al. 2001). PEP carboxylase induction in roots is paralleled by increased levels of several glycolytic enzymes, namely, Ga3PDHase, phosphoglyceromutase, enolase, and PyrKase. This, together with an enhanced mitochondrial electron transport complement, reveals a respiratory surge as a response to iron stress.

#### 18.4.2 SOLUBLE CARBOHYDRATES AND THEIR ROLE AGAINST STRESS

Many organisms accumulate low molecular weight compounds such as disaccharides (sucrose and trehalose), sugar-alcohols, quaternary amines, or amino acids. It has been proposed that these molecules allow organisms tolerate certain kinds of abiotic stresses (like salinity, cold or drought) through mass action. These compounds may be considered as compatible solutes, a term that was introduced in studies conducted with yeast accumulating nonreducing sugars (like trehalose) and sugar-alcohols (including glycerol) in response to osmotic stress (Brown and Simpson 1972). It is well known that different inorganic ( $K^+$ ,  $Na^+$ ,  $Cl^-$ , and  $SO_4^{2-}$ ) and organic (reducing hexoses) molecules are critical to establish osmotic adjustments in aqueous systems. However, these compounds are different from compatible solutes, which may affect the properties of solutions by distinctive ways (Bohnert and Jensen 1996, Loescher and Everard 2004). Early work established that high concentrations of compatible solutes do not interfere with *in vitro* enzyme activities and that sometimes they can protect proteins from deleterious effects of salts or heat (Bohnert and Jensen 1996, Loescher and Everard 2004). It has been described that the concentration of these molecules should reach values as high as 500 mM to exert protection; interestingly, such levels may be achieved in cells (Moing et al. 1997, Nadwodnik and Lohaus 2008).

Different explanations have been proposed to clarify the protective effect of compatible solutes on biological structures. One hypothesis is that they substitute water molecules in the hydration of proteins and membranes, thus allowing enzyme activity to occur even at extremely low water concentrations (Webb and Bhorjee 1968). Another alternative suggests that compatible solutes might be outside the hydration sphere of proteins, thus producing a particular rearrangement of the sphere and inducing the biological structure to adopt a preferential hydration (Timasheff 1993). Concerning plants, studies performed at the present time highly support the possibility to derivate cytosolic carbohydrates to different metabolic fates rendering compounds of the type of compatible solutes. Accumulation of these metabolites could help the plant to resist extreme conditions of low temperatures, high salt levels, and water deficit. The overall picture sustain that modifications in the balance of photosynthates partitioning can play critical roles for plant productivity and survival under physiological or stress conditions.

##### 18.4.2.1 Osmotic Stress Adaptation

Plants response to osmotic stress generally results in increased soluble sugars and decreased starch as a result of enhanced sugar synthesis. This rise in carbohydrates concentration has been related with higher activity levels of the synthesizing enzymes. For instance, SPSase activity dramatically increased in osmotically stressed spinach leaves and potato tubers. This activation is consequence of the phosphorylation of a single residue (serine-424 in spinach leaf SPSase), and is different from that involved

in dark-light modulation (Winter and Huber 2000). This site is widely conserved among species and its phosphorylation in osmotically stressed leaves activates the enzyme, thus allowing sucrose synthesis to occur when it would otherwise be restricted (Toroser and Huber 1997). Accumulation of sucrose, cyclic, or acyclic sugar-alcohols, proline, and quaternary amines (like glycinebetaine) could potentially play a direct role in osmoregulation and could also provide quickly metabolizable carbohydrates for energy production when carbon is diverted from growth to other functions (Hare et al. 1998).

#### 18.4.2.2 Low-Temperature Stress

Carbohydrates such as sucrose, fructans, and sugar-alcohols can be important in tolerance and resistance to cold-induced damage (del Viso et al. 2009a,b, Loescher and Everard 2004, Pontis 1989, Tognetti et al. 1990). Cold damage results by desiccation due to water demand from the protoplast, as a consequence of the growing ice crystal (Loescher and Everard 2004). Even when results are not conclusive, many studies have related this kind of stress with the accumulation of compatible solutes. For instance, it was found that Gol concentration in apple shoot xylem increased with leaf senescence and low temperatures (Williams and Raese 1974). Similar changes have been described in plum trees, where the highest level of Gol in sap was found after exposing plants to temperatures below zero (Loescher et al. 1990). Further evidence in the same way was obtained by Hirai (1983), who reported a raise of Gol and A6PRase levels in loquat leaves during low-temperature seasons. In addition, accumulation of sucrose in photosynthetic and heterotrophic tissues has been linked with increased SPSase activity under nonfreezing temperatures, which was related to an increased level of SPSase protein in spinach leaves (Guy et al. 1992) and potato tubers (Hill et al. 1996). In spinach leaves, the rate of SPSase protein synthesis seems to be responsible for the raise in activity and the newly produced enzyme subunit appears to be identical to that found under normal conditions (Guy et al. 1992). On the other hand, cold-exposed potato tubers showed an increase in a particular subunit (1b) of SPSase, which correlates with a change in the kinetic properties of this enzyme. Thus, the altered kinetics of SPSase may play an important role in the regulation of sucrose synthesis in cold-stored tubers (Hill et al. 1996).

#### 18.4.2.3 Drought Stress

Results relating drought stress and carbohydrates accumulation are difficult to understand as a result of secondary effects, like growth inhibition and dehydration (Loescher and Everard 2004). However, it is important to draw attention to the evidences. For instance, Gol was the main soluble carbohydrate and its content was doubled in plants from the *Prunus* genus subjected to drought stress (Ranney et al. 1991). In apple trees, drought stress resulted in the preferential accumulation of Gol and Glc at the expense of sucrose and starch (Wang et al. 1995, 1996). When genetically transformed plants were used, it was demonstrated that the increased ability for synthesizing soluble sugar-alcohols promoted drought tolerance. In transformed tobacco plants synthesizing the nonreducing disaccharide trehalose, it was determined that their ability to survive after a drought period was enhanced. However, their phenotypes were altered and the growing rate was diminished up to 50% under normal growing conditions (Holmstrom et al. 1996). A similar work, where transformed tobacco plants were engineered to produce bacterial fructans, showed that these plants grew better than controls under polyethyleneglycol-induced drought, conditions where the growing rates and the fresh and dry weights were also higher. On the other hand, compared with those plants producing trehalose, these transformed plants did not show differences with controls under normal conditions (Pilon-Smits et al. 1995).

#### 18.4.2.4 High-Salinity-Induced Stress

The correlation between salt stress and sugars accumulation is quite strong, and these works represent the most numerous reports linking one type of stress with polyols accumulation. A couple of studies, done with celery exposed to NaCl-induced stress (Everard et al. 1994) or macronutrients excess (Stoop and Pharr 1994) established that changes in mannitol metabolism were consequence of both carbon partitioning and utilization. For instance, in newly developed celery leaves exposed to 300 mM NaCl, the carbon flux into mannitol remained at similar rates to controls, although a 70%

decrease in carbon assimilation was found. The maintenance of mannitol synthesis was at sucrose expenses, thus increasing fourfold the ratio between mannitol and sucrose labeled. This change was associated to an increase in the activity of mannose-6-P reductase (EC 1.1.1.224). However, this increase in activity was not related to a rise in the protein level of the enzyme, suggesting a possible posttranslational modification of the enzyme (Everard et al. 1994). On the other hand, a study conducted with Japanese persimmon (*Diospyros kaki*, a plant that normally does not produce sugar-alcohols) transformed with the gene encoding for the A6PRase from apple leaves, found an increase in tolerance to NaCl-induced stress in those lines capable of accumulating Gol (Gao et al. 2001).

#### 18.4.2.5 Hydroxyl-Radical Scavengers

Reduced stomatal conductance can occur under salt and drought stress, thus increasing the production of free radicals. There is evidence that in fungi and other organisms sugar-alcohols (such as Gol and mannitol) and cyclic polyols (like *mio*-inositol) can act as free radical scavengers (Jennings and Burke 1990, Smirnoff and Cumbes 1989). Also, it was shown that this effect could be observed in stress caused by dehydration (Smirnoff 1993). However, the evidence of *in vivo* assays is limited. For instance, transformation of tobacco plants with a bacterial mannitol-1-P dehydrogenase (EC 1.1.1.17), directed to chloroplasts by the introduction of a transit peptide, rendered a line capable of accumulating mannitol in chloroplasts in concentrations up to 100 mM, with no alteration of the phenotype or the photosynthetic activity. The presence of mannitol in chloroplasts resulted in an increased tolerance to methylviologen, a compound that produces oxidative stress. It is important to emphasize that the presence of the polyol did not reduce the abundance of reactive oxygen species, but it conferred an additional protection to the already present in non-transformed plants (Shen et al. 1997).

### 18.5 CONCLUDING REMARKS

As can be inferred from the numerous examples listed above, carbon metabolism and partitioning critically affect plant productivity under physiological and stress conditions. Thus, the understanding of key enzymes involved in different metabolic routes operating in plants is relevant to evaluate potential plant adaptation in different environments. Many factors can affect the expression pattern of genes and the activity of enzymes related to carbohydrates metabolism. In general, the modulation of enzyme activity by metabolites (allosterism) or posttranslational modifications (thiols oxidation and reduction or phosphorylation) have been relatively well established in sucrose and starch biosynthetic pathways. On the other hand, the enzymes involved in sugar-alcohols metabolism have been scarcely characterized. Thus, efforts should be made to develop accurate systems to express and purify these enzymes, and such a work is currently under way (Figueroa and Iglesias 2010, Ohta et al. 2005). Developing molecular tools to identify the functionality of different enzymes and to rationally modify plant metabolism is promissory to handle plant behavior in diverse habitats.

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# 19 Protein Synthesis by Plants under Stressful Conditions

*Pallavi Sharma and Rama Shanker Dubey*

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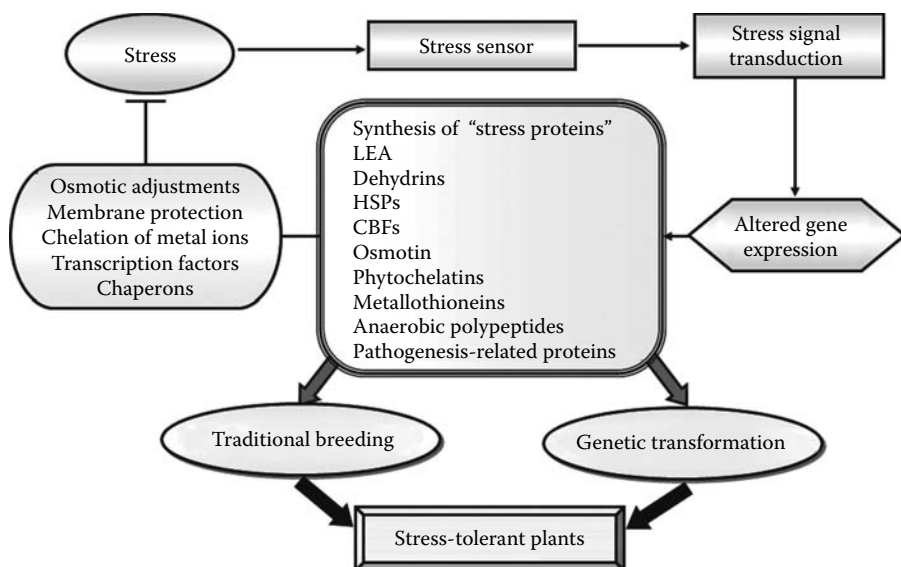
## 19.1 INTRODUCTION

Environmental stresses present major challenges in our quest to achieve sustainable food production. The reactions of plants to environmental stresses are complex and involve a wide array of physiological and biochemical responses. Such responses are initiated by plants growing in stressed environments to overcome, avoid, or nullify the effects of stresses. Tolerance or sensitivity toward a particular stressful condition depends on the genetic and biochemical makeup of the species. Much attention has been focused during recent years to evolve crop species with adaptability built into their genetic and biochemical makeup to withstand various stressful environmental conditions.

Plants are unable to express their full genetic potential for production when subjected to stressful environments (Zeigler, 1990; Gao et al., 2007). When exposed to stressed environments, plants initially recognize the stress stimulus, and thereafter a signal transduction cascade is

invoked. Secondary messengers relay the signal, activating stress-responsive genes, the expression of which causes the accumulation or depletion of certain metabolites, alteration in the activity behaviors of many enzymes, overall changes in protein synthesis, and, of particular interest, synthesis of new sets of proteins that are specific to the particular type of stress (Jacobsen et al., 1986; Vierling, 1991; Kaur and Gupta, 2005; Sharma and Dubey, 2007; Kumar et al., 2008; Gupta et al., 2009). It has been shown that different environmental stresses induce the synthesis of novel proteins in plants, which possibly provide evolutionary value to the plants for enhanced survival in adverse environmental situations. The synthesis of such stress-induced proteins has been well documented under salinity stress (Ericson and Alfinito, 1984; Hurkman and Tanaka, 1987; Singh et al., 1987; Ben-Hayyim et al., 1989; Naot et al., 1995; Naqvi et al., 1995; Igarashi et al., 1997; Aarati et al., 2003; Mahmoodzadeh, 2009), osmotic stress or drought (Bewley and Larsen, 1982; Singh et al., 1987; Vance et al., 1990; Robertson and Chandler, 1994; Baker et al., 1995; Mantyla et al., 1995; Perezmolphebalch et al., 1996; Zhang et al., 1996; Jiang and Huang, 2002; Demirevska et al., 2008; Khurana et al., 2008), heat shock (Heikkila et al., 1984; Mansfield and Key, 1988; Vierling, 1991; Cordewener et al., 1995; Waters et al., 1996; Lee et al., 2007; Yildiz and Terzi, 2008), low-temperature treatment (Meza-Basso et al., 1986; Hahn and Walbot, 1989; Bruggemann et al., 1994; Griffith et al., 1997; Matsuba et al., 1997; John et al., 2009; Kikuchi and Masuda, 2009), anaerobiosis (Ricard et al., 1991; Christopher and Good, 1996; Sachs et al., 1996; Subbaiah and Sachs, 2003), infection with pathogens (Antoniw et al., 1980; Ohashi and Matsuoka, 1985; Abad et al., 1996; Herbers et al., 1996; Tornero et al., 1997; Almagro et al., 2009), wounding (Cabello et al., 1994; Jung et al., 1995; Schaller and Ryan, 1996; Jimenez et al., 2008; Dafoe et al., 2009), metal toxicity (Choi et al., 1995; Shah and Dubey, 1998a; Sharma and Dubey, 2007), gaseous pollutants (Kirtikara and Talbot, 1996), and ultraviolet (UV) radiation (Jung et al., 1995; Rao et al., 1996; Xu et al., 2008; Pan et al., 2009). Figure 19.1 shows the schematic diagram of synthesis of proteins under different stresses in plants. These proteins, which are stress specific, present newer avenues to improve stress tolerance of plants.

The main idea underlying studies of stress-induced synthesis of proteins in plants is that the different sources of stresses, their duration, and severity lead to a differential expression of genetic



**FIGURE 19.1** Stress-induced protein synthesis in plants. Stresses cause important modifications in the gene expression in plants, which leads to the synthesis and accumulation of stress-related proteins. These proteins provide enhanced survival value to plants under adverse environmental situations and can be used to produce stress-tolerant plants by genetic transformations. For details, refer Section 19.2.

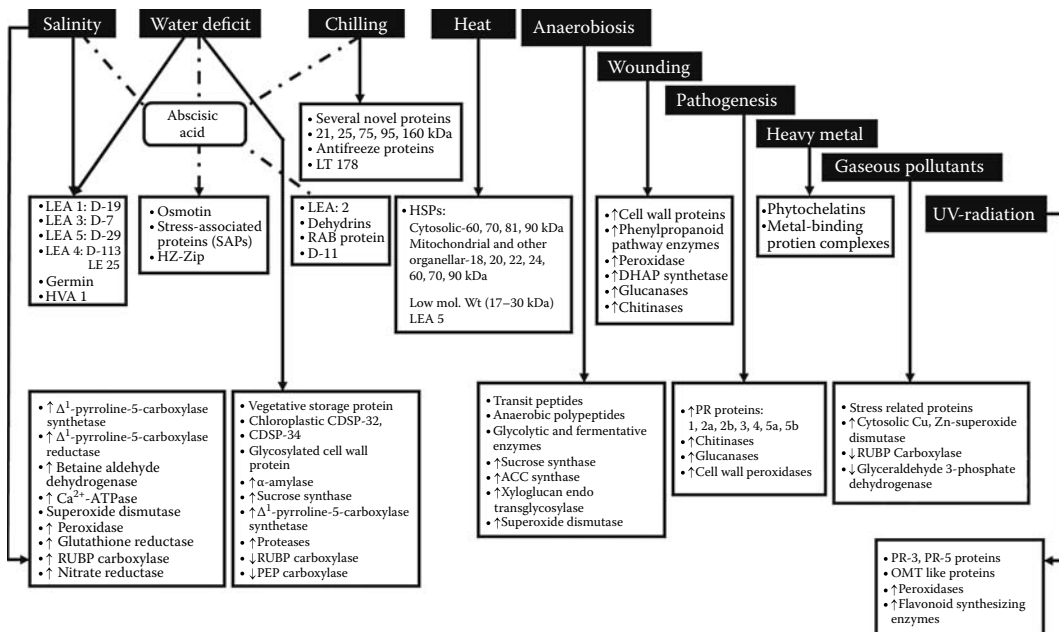
information, resulting in changes in gene products, including mRNA and proteins. Such newly synthesized proteins are specific to the particular type of stress and possibly confer enhanced survival value to the plants (Ben-Hayyim et al., 1989). Stress-induced proteins identified from different organs of plants have been well characterized. Physicochemical parameters such as molecular weight and pI (isoelectric point) values of these proteins have been deduced (Ben-Hayyim et al., 1989; Robertson and Chandler, 1994; Sachs et al., 1996; Waters et al., 1996; Efeoglu and Terzioglu, 2007), and, in many cases, data regarding association characteristics and amino acid sequences have also been reported (Singh et al., 1987; Badur et al., 1994; Naot et al., 1995; Zhang et al., 1996). A sizeable volume of literature indicates quantitative and qualitative changes in proteins, when plants are stressed, mainly employing the methods of electrophoresis, the western analysis, enzyme kinetics studies, etc. Nowadays, high-throughput stress-induced protein identification and characterization systems using tools of proteomics are available. Improved protein extraction and purification protocols have been devised, and genomic sequence databases for peptide mass matches are available (Timperio et al., 2008). Although the stress proteins are synthesized in plants when they are subjected to stresses and can be revealed in tissues of plants adapted to stress, specific metabolic functions for most of these proteins have not been established as to how they confer adaptability toward stress (Ben-Hayyim et al., 1989; Artlip and Funkhouser, 1995; Khurana et al., 2008). Particularly, under anaerobic stress, the polypeptides that are synthesized have specific functions and belong to the enzymes of sugar phosphate metabolism (Ricard et al., 1991). Heat-shock proteins (HSPs), which are synthesized under heat stress, possibly assist in protein folding, protein-protein interactions, and the translocation of proteins across cellular compartments, and they have a possible role in protecting the organism from heat stress (Cordewener et al., 1995; Wang et al., 2004). Similarly, the pathogenesis-related (PR) proteins do act in the defense of the plants and have a putative role in pathogen resistance (Artlip and Funkhouser, 1995). Under salinity stress, it is suggested that the newly synthesized proteins, together with amino acids and soluble nitrogenous compounds, act as components of a salt-tolerance mechanism. These might function as compatible cytoplasmic solutes in osmotic adjustment in order to equalize the osmotic potential of the cytoplasm with the vacuoles in adverse conditions of salinity (Greenway and Munns, 1980; Dubey and Rani, 1989).

Studies related to stress-induced synthesis of proteins have been performed using cultured plant cells (Ericson and Alfinito, 1984; Singh et al., 1987; Ben-Hayyim et al., 1989; Vance et al., 1990; Sobkowiak and Deckert, 2006), seedlings (Mansfield and Key, 1988; Hahn and Walbot, 1989; Han and Kermode, 1996; Igarashi et al., 1997; Efeoglu and Terzioglu, 2007), excised plant organs (Hurkman and Tanaka, 1987; Stuiver et al., 1988), and intact plants (Burke et al., 1985; Ohashi and Matsuoka, 1985; Kee and Nobel, 1986; Popova et al., 1995). Among these systems, cultured plant cells have proven to be superior to other systems as they show uniform response and are under better control of environmental parameters (Ben-Hayyim et al., 1989; Fadzilla et al., 1997). Cell cultures from tobacco, cowpea, potato, citrus, and many other plant species have been used to identify and characterize newly synthesized proteins under salinity, heat-shock, freezing, osmotic, and heavy metal stresses (Singh et al., 1987; Ben-Hayyim et al., 1989; Vierling, 1991; Naot et al., 1995; Fadzilla et al., 1997). During recent years, a wealth of literature has been available, dealing with environmental stress-induced proteome changes, based on whole tissue/organ analysis (Bae et al., 2003; Giacomelli et al., 2006; Goulas et al., 2006; Taylor et al., 2009). Taylor and coworkers (2009) analyzed data and reviewed a collection of both whole tissue and organellar proteomic studies that investigated the effects of environmental stress in the model plant *Arabidopsis thaliana*. They found 279 proteins that could change in abundance and could be assigned as protein components of the energy organelles (chloroplast, mitochondria, and peroxisomes). These could be placed into eight different functional categories, and nearly 80% of the specific protein isoforms detected were only reported to change in a single environmental stress.

Besides, the identification of specific-stress-induced proteins, several investigators have tried to quantify the overall metabolic status of total and soluble proteins (including enzymes) of different metabolic pathways in stressed plant parts in order to evaluate the impact of stresses on various aspects

of plant growth and metabolism (Dubey and Rani, 1987; Dubey and Rani, 1989; Gogorcena et al., 1995; Shah and Dubey, 1995a,b; Jha and Dubey, 2004a,b; Mishra and Dubey, 2006, 2008a,b; Sharma and Dubey, 2007; Maheshwari and Dubey, 2007, 2008, 2009). Environmental stresses generally are detrimental to plant growth, adversely affect the metabolism of plants, and cause an imbalance in the level of protein as a result of their effects on the synthesis and hydrolysis of proteins (Dubey and Rani, 1987; Elsamad and Shaddad, 1997; Moons et al., 1997; Shah and Dubey, 1998a,b; Mishra and Dubey, 2006; Maheshwari and Dubey, 2007, 2008). Abiotic stresses lead to an enhanced degradation of proteins. Amino acids derived from protein catabolism may be redistributed within the plant via the phloem and serve as a basis for protein synthesis in other plant parts (Feller et al., 2008). In salt- and drought-stressed plant parts, the protein content decreases owing to the decreased rate of protein synthesis and the increased rate of proteolysis (Dubey and Rani, 1987, 1990; Perezmolphebaltch et al., 1996; Sharma and Dubey, 2005a). In seeds germinating under salinity, moisture stress, or nickel toxicity, however, an increase in the protein level is observed. This increase can best be explained by the fact that in germinating seeds, stress causes decreased proteolysis in endosperms, resulting in a slower depletion of reserve proteins. This reflects an apparent increase in the endospermic protein level under stress, which is not a result of enhanced protein synthesis (Dubey, 1983a; Dubey and Rani, 1987, 1990; Maheshwari and Dubey, 2008). Salt tolerance is dependent on the genetic and biochemical characteristics of the species. Therefore, attempts have been made by certain groups of investigators to differentiate stress-tolerant and stress-sensitive genotypes of crops on the basis of profiles or levels of soluble proteins, specific enzymes in germinating seeds, and growing plant parts (Dubey and Rani, 1987, 1989; Perezmolphebaltch et al., 1996; Elsamad and Shaddad, 1997; Mahmoodzadeh, 2009). The results of these attempts indicate that different levels of soluble proteins and many enzymes exist in the two sets of genotypes differing in stress tolerance.

Studies conducted so far indicate that stressful conditions adversely affect the protein metabolism in plants and that in all different types of environmental stresses, such as salinity, drought, heat, chilling, anaerobiosis, pathogenesis, wounding, heavy metal toxicity, and gaseous



**FIGURE 19.2** An overview of stress-induced protein-synthetic responses in plants. Different stresses induce the synthesis of various groups of proteins and cause either elevation (↑) or decline (↓) in the levels of enzymes. Some of the responses of salinity, drought, and chilling are common and are mediated via elevated levels of ABA. For details, refer Section 19.1.

pollutants, new stress-specific proteins are synthesized. These proteins might play a role in signal transduction, antioxidative defense, antifreezing, heat shock, metal binding, anti-pathogenesis, or osmolyte synthesis (Qureshi et al., 2007; Timperio et al., 2008). Recent developments in sensitivity and accuracy for proteome analysis have provided new dimensions in identifying novel proteins and in assessing the changes in protein types and their expression levels under stresses (Qureshi et al., 2007). An overview of protein-synthetic responses in plants and alterations in the levels of key enzymes under various stresses is presented in Figure 19.2. These proteins that have been discussed in detail in Section 19.2 can be successfully used as attractive targets to produce stress-tolerant plants using biotechnological approaches (Table 19.1). The identification of novel stress-responsive proteins provides not only new insights into stress responses but also a good starting point for further dissection of their functions (Yan et al., 2006), and opens up new avenues for the production of stress-tolerant plants using traditional breeding or transgenic approaches. This chapter presents our current status of knowledge related to the effect of various environmental stresses on the overall aspects of protein synthesis in plants, and the possible role of stress-specific proteins in conferring an enhanced survival value to the plants against various environmental stress situations.

**TABLE 19.1**  
**Transgenic Stress-Tolerant Plants Produced by Using Stress-Related Proteins**

| Stress Proteins    | Gene                | Stress              | Plant                 | References                                 |
|--------------------|---------------------|---------------------|-----------------------|--------------------------------------------|
| Antifreeze protein | <i>AFP</i>          | Freezing stress     | Tobacco               | Worrall et al. (1998)<br>Fan et al. (2002) |
| LEA proteins       | <i>BnLEA4-1</i>     | Drought             | <i>Arabidopsis</i>    | Xu et al. (1996)                           |
|                    | <i>HVA1</i>         | Salt                | Rice                  | Hu (2008)                                  |
|                    | <i>OsLEA-3</i>      |                     | Tobacco               | Dalal et al. (2009)                        |
|                    | <i>LEA 4</i>        |                     |                       | Liu et al. (2009)                          |
| Dehydrins          | <i>Dhn5</i>         | Salinity            | <i>Arabidopsis</i>    | Puhakainen et al. (2004)                   |
|                    | <i>Dhn24</i>        | Osmotic stress      | Cucumber              | Yin et al. (2006)                          |
|                    | <i>RAB18</i>        | Chilling            |                       | Brini et al. (2007)                        |
|                    | <i>COR47</i>        |                     |                       |                                            |
|                    | <i>LTI29</i>        |                     |                       |                                            |
| HSPs               | <i>LTI30</i>        |                     |                       |                                            |
|                    | <i>sHSP17.7</i>     | Drought             | Rice                  | Sato and Yokoya (2008)                     |
|                    | <i>NtHSP70-1</i>    | Salt                | Tobacco               | Cho and Choi (2009)                        |
|                    | <i>AtHsp90.2</i>    | Heat stress         | <i>Arabidopsis</i>    | Song et al. (2009)                         |
|                    | <i>AtHsp90.5</i>    |                     |                       |                                            |
| CBF/DREB           | <i>AtHsp90.7</i>    |                     |                       |                                            |
|                    | <i>DREB1A</i>       | Drought             | Tobacco               | Oh et al. (2007)                           |
|                    | <i>OsDREB1B</i>     | Freezing stress     | <i>Paspalum</i>       | Gutha and Reddy (2008)                     |
|                    | <i>HvCBF4</i>       |                     | <i>notatum</i> Flügge |                                            |
| Osmotin            |                     | High-salinity       | Rice                  | James et al. (2008)                        |
|                    | <i>Osmotin gene</i> | Salinity            | Cotton                | Barthakur et al. (2001)                    |
|                    |                     | Drought             | Tobacco               | Husaini and Abidin (2008)                  |
|                    |                     |                     | Strawberry            | Parkhi et al. (2009)                       |
| PC                 | <i>AtPCS1</i>       | Arsenic             | Indian mustard        | Pomponi et al. (2006)                      |
|                    | <i>p5cs</i>         | Cadmium             | Tobacco               | Gasic and Korban (2007)                    |
| MT                 | <i>BrMT1</i>        | Cadmium             | <i>Arabidopsis</i>    | An et al. (2006)                           |
|                    | <i>BjMT2</i>        | Copper              |                       | Kim et al. (2007)                          |
| PR proteins        | <i>OgChitIVa</i>    | <i>B. cinerea</i>   | <i>Arabidopsis</i>    | Velazhahan and                             |
|                    | <i>PR-5</i>         |                     |                       | Muthukrishnan (2003)                       |
|                    |                     | <i>A. alternata</i> | Tobacco               | Pak et al. (2009)                          |

## 19.2 STRESSED ENVIRONMENTS AND PROTEIN SYNTHESIS

The major type of stresses to which plants are exposed include salinity, drought, flood, heat, cold, anaerobiosis, infection by pathogens, metal toxicity, gaseous pollutants, and UV radiation. Plant metabolism and, more specifically, protein synthesis are adversely affected under these conditions. The effect of stress depends on the developmental stage of the plant, genotypes of the plant species, as well as the intensity and duration of the stress. The progress achieved so far in our understanding of the impact of different environmental stresses on protein synthesis is described in Sections 19.2.1 through 19.2.10.

### 19.2.1 SALINITY

Soil salinity is a major environmental stress that drastically affects crop productivity. Salinity poses a severe threat for the cultivation of crops in arid and semiarid regions of the world. Due to the continuous buildup of salinity in the soil, millions of hectares of usable land have now become unsuitable for cultivation. It is estimated that every year more than a million hectares of land is subjected to salinization. Soil salinity is thus threatening the civilization by persistently reducing the area for crop cultivation. Salinity not only causes great losses in crop yields but also has an impact on other economic, environmental, social, and political problems in the affected countries. The progress in developing salt-tolerant crop varieties has been very slow due to our incomplete knowledge of the mechanism of salt damage and the complex nature of salt tolerance. Even different varieties of a particular species may exhibit different tolerance behaviors. Salinity affects seed germination, plant growth, nutrient uptake, and metabolism owing to osmotic inhibition of water availability, ion imbalance, toxic effects of salt ions, and their effects on cellular gene expression machinery (Dubey and Pessarakli, 1995; Abdelkader et al., 2007). Different plant species have developed different mechanisms to cope up with salinity stress effects (Munns, 2002; Abdelkader et al., 2007). Salinity promotes the synthesis of salt stress-specific proteins (Hurkman and Tanaka, 1987; Singh et al., 1987; Ben-Hayyim et al., 1989; Artlip and Funkhouser, 1995; Mahmoodzadeh, 2009); causes either decreases (Popova et al., 1995) or increases (Dubey, 1983a; Elenany, 1997) in the level of total and/or soluble proteins, depending on the plant parts studied; and leads to increased activity/synthesis of many enzymes (Dubey and Rani, 1987, 1990; Dubey et al., 1987; Mittal and Dubey, 1991, 1995; Igarashi et al., 1997).

#### 19.2.1.1 Salt-Induced Protein Synthesis

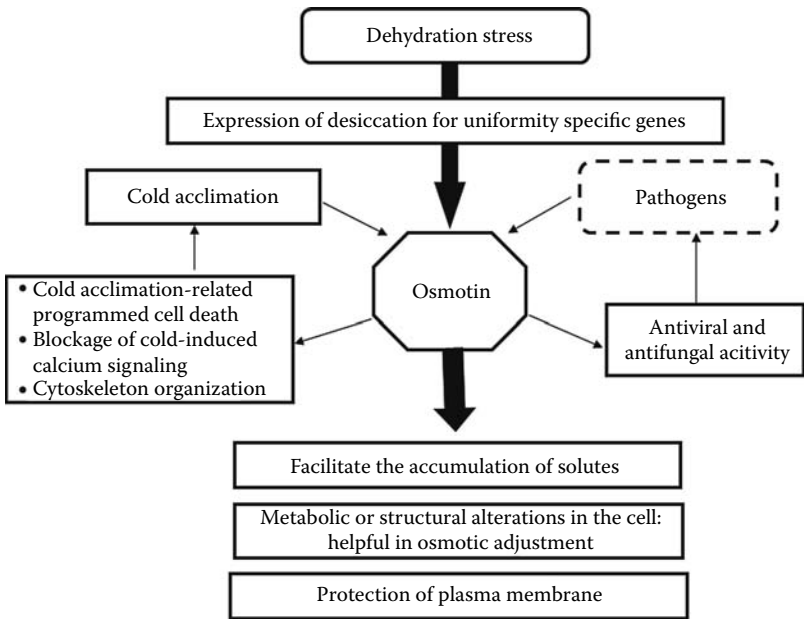
Plants growing in a saline environment show distinct changes in the pattern of synthesis and accumulation of proteins. Most of the experiments to study the salinity-induced synthesis of proteins have been conducted using plant cell cultures. Cell cultures rather than whole plant systems have proven to be more advantageous for such studies, because, in cell cultures, environmental parameters can be better controlled and the stress-tolerant cell lines generated can be readily selected and assayed for newly synthesized proteins.

Several investigators have shown the synthesis of new proteins in cultured plant cells when subjected to salinity stress (Ericson and Alfinito, 1984; Singh et al., 1987; Ben-Hayyim et al., 1989; Artlip and Funkhouser, 1995; Elenany, 1997). The level of proteins differs in salt-tolerant and salt-sensitive genotypes when they are subjected to salinity stress (Dubey and Rani, 1989; Elsamad and Shaddad, 1997; Mahmoodzadeh, 2009; Tada and Kashimura, 2009). Although it is well established that salt tolerance and sensitivity depend on the genetic and biochemical compositions of the species, it has been difficult so far to specify the exact genetic domain responsible for salt-adaptation expression leading to the synthesis of these proteins in salt-adapted plants. These specifically synthesized proteins under salt stress appear to have a role in providing tolerance or adaptation to the plants. However, the overall mechanism of how these proteins could provide adaptation is not clearly understood.



To understand the mechanism of salt resistance in cultured tobacco cells, Ericson and Alfinito (1984) examined the protein patterns of NaCl-adapted as well as NaCl-nonadapted cell lines of tobacco (*Nicotiana tabacum* L.). Their results indicated that cells adapted to a medium containing NaCl showed two protein bands of 32 and 20 kDa in more abundance than unadapted cells. Further, in the salt-adapted cells, a unique protein of 26 kDa appeared that was specific for these cells and was not present in unadapted cells or cells growing without NaCl. These investigators suggested that the three proteins synthesized in salt-adapted cells might be involved in a salt-adaptation process. Yildiz (2007) observed the synthesis of two new low-molecular-weight (LMW) proteins (28.9 and 30.0 kDa) and one intermediate-molecular-weight (IMW) protein (44.3 kDa) in response to the NaCl treatment in wheat cv. Ceyhan-99 (salt sensitive), whereas six LMW proteins (18.6, 19.4, 25.7, 25.9, 26.0, and 27.6 kDa) were newly synthesized in wheat cv. Firat-93 (salt tolerant). The newly synthesized proteins were specific to each cultivar. Most of the newly synthesized proteins were acidic in nature with mol. wt. <31.6 kDa. These newly synthesized proteins were suggested to be important for cultivars differing in sensitivity toward NaCl (Yildiz, 2007).

According to Singh et al. (1985), in cultured tobacco cells, the process of cellular adaptation to osmotic stress in a saline environment involves the specific alteration in the gene expression of salt-adapted cells, leading to the synthesis of several novel proteins, including the predominant 26 kDa protein. Since the 26 kDa protein is specifically synthesized and accumulated in cells undergoing an osmotic adjustment to salt or dessication stress, this protein has been named “osmotin” (Singh et al., 1987). Plant cells undergoing dessication stress specifically synthesize and accumulate osmotin, which provides the osmotic adjustment to the cell. Osmotin also plays a role in cold acclimation (CA) and in response against viral and fungal pathogen infections (Figure 19.3). Osmotin is regarded as a unique protein associated with NaCl-adapted tobacco cells (Singh et al., 1987). Interestingly, the synthesis of osmotin is not induced by osmotic shock but starts only when cells are adapted to NaCl or polyethylene glycol (PEG) (Singh et al., 1985). In salt-adapted cells, osmotin constitutes about 10%–12% of the total protein content in the cell (Singh et al., 1985). Tada and Kashimura (2009) observed an increased osmotin content at early time points following salt treatment in lateral



**FIGURE 19.3** Plant cells undergoing dessication stress specifically synthesize and accumulate osmotin, which helps in providing osmotic adjustment to the cell. Osmotin also plays a role in CA and against viral and fungal pathogen infections.

roots of the mangrove plant *Bruguiera gymnorhiza*, and concluded that osmotin may play a role in the initial osmotic adaptation in lateral roots of *B. gymnorhiza* under salt stress, but does not contribute toward adaptation to prolonged or continuous exposure to salt stress. A primary role played by osmotin during low water potential conditions appears to be as a protectant of the plant plasma membranes (PMs) (Singh et al., 1989).

It is believed that osmotin plays the role of providing an osmotic adjustment to the cells either by facilitating the accumulation of solutes or by providing certain metabolic or structural alterations in the cell, which may be helpful in osmotic adjustment (Singh et al., 1987). Although some understanding of the possible mechanism underlying the defense function of osmotin against biotic stresses is beginning to emerge, its role in stress response is far from clear (Parkhi et al., 2009). Protective abilities of osmotin against salinity stress have been confirmed by overexpression studies. The constitutive expression of an osmotin gene in transgenic tobacco was found to improve its tolerance to salinity (Barthakur et al., 2001). Improved germination of seeds and higher growth rates of plants under high salinity were observed in transgenic strawberry expressing tobacco osmotin (Husaini and Abdin, 2008).

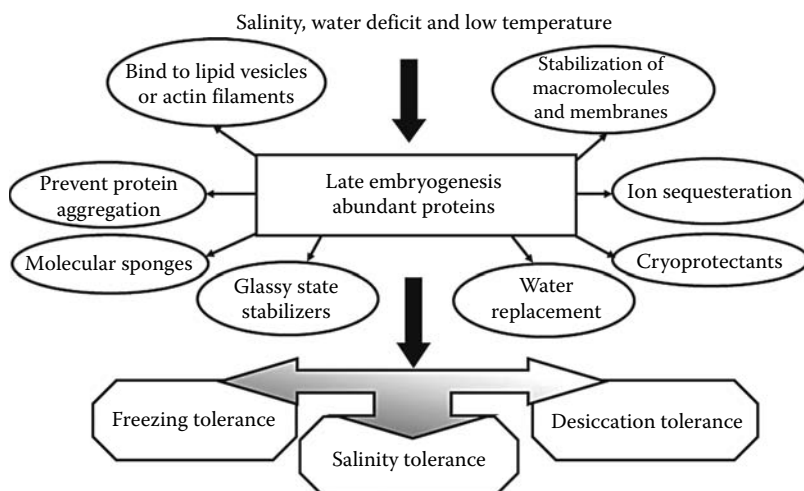
Similar to tobacco cells, the synthesis of salt-induced proteins has also been shown in barley (Hurkman and Tanaka, 1987; Popova et al., 1995), maize (Ramagopal, 1986), citrus (Ben-Hayyim et al., 1989; Naot et al., 1995), rice (Naqvi et al., 1995), and finger millet (Uma et al., 1995). Barley plants, when subjected to a short-term NaCl shock or a long-term NaCl treatment for a period of 8 days show marked quantitative and qualitative changes in protein profiles compared to nonstressed plants (Hurkman and Tanaka, 1987; Popova et al., 1995). The maize callus tissue (Ramagopal, 1986) shows predominantly the accumulation of a 26 kDa protein under saline stress. In potato plants, high salinity leads to an increased synthesis of 32 and 34 kDa proteins in the thylakoids (Pruvot et al., 1996). A comparison of the protein profiles of nonadapted and NaCl-adapted cell lines of citrus and tomato indicates that, in citrus, the level of most proteins is suppressed, whereas, in tomato, it is enhanced under salt stress (Ben-Hayyim et al., 1989). Tomato cell cultures when grown in a medium with 25 mM NaCl and proline, synthesize extra polypeptides of 190, 58, 45, and 26 kDa, and with the further increase of NaCl in the medium, a new 67 kDa protein gets accumulated (Elenany, 1997). In tobacco cells, an enhancement in the level of certain proteins and a decrease in the level of others are observed when cells are adapted to NaCl (Singh et al., 1987). This indicates that salt-induced changes in proteins are species specific and that different proteins are associated with salt tolerance in different species. Using germinating seeds of broad bean (*Vicia faba*) lines, Ahmed and coworkers (2001) showed that salinity stress caused an increase in the number of protein bands in bean line 67 (salt tolerant), whereas no considerable effect of salinity stress on the protein patterns could be observed in bean line 13 (salt sensitive).

An electrophoretic analysis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) of total soluble protein profiles of canola salt-tolerant cv. Okapi and salt-sensitive cv. Symbol in response to salt stress showed induction in the synthesis of few polypeptides in seeds. The number of induced proteins was greater in the tolerant cultivar than in the sensitive. These differences could be associated with the biochemical adjustment of the plants to cope up with the saline conditions (Mahmoodzadeh, 2009). In certain plant species, such as rice (*Oryza sativa* L.) and Shamouti orange (*Citrus sinensis* L. Osbeck), it has been shown that salt-sensitive and salt-tolerant genotypes have different patterns of protein profiles whether grown in the absence or the presence of NaCl. Salt-tolerant genotypes of rice possess a 28 kDa protein in shoots, which is absent in salt-sensitive genotypes, and the level of this protein is further elevated when the rice seedlings are raised in a saline medium (Rani, 1988). This shows that the presence of the additional 28 kDa protein band is associated with salt-tolerance characteristics in rice. In salt-sensitive rice species, some of the major preexisting proteins disappear and certain new proteins appear with an increase in salinity. Ten-day-old seedlings of rice cv. Nonabokra, Basmati, IR 28, and IR 29 when treated with 2% NaCl for 8 h show a synthesis of two new proteins of 27.0 and 25.5 kDa (Naqvi et al., 1995), whereas the seedlings of certain rice cultivars when exposed to salinity stress accumulate 87 and

85 kDa stress-associated proteins (SAPs) (Pareek et al., 1997). Similar to rice, in citrus (*C. sinensis* L. Osbeck), a 25 kDa protein has been shown to be associated with salt tolerance (Ben-Hayyim et al., 1989). This protein appears to be a constitutive protein in salt-tolerant citrus cells and is present whether cells are grown in the absence or the presence of NaCl. The level of this protein is enhanced when cells are grown in a saline medium. An enhancement can be readily observed by growing the cells in a growth medium containing 1% NaCl.

Since the synthesis of constitutive proteins associated with salt tolerance in rice or citrus does not depend on the presence of salt in the growth medium, it appears that the salt-tolerance trait is stable in these species. Salt-tolerant lines in these species show a synthesis of such constitutive proteins up to many generations when grown either in the presence or in the absence of NaCl. Similar constitutive proteins have also been shown to be associated with sensitive or tolerant genotypes of finger millet. Among finger millet (*Eleusine coracana* Gaertn.) genotypes differing in tolerance to NaCl, a 200 mM the NaCl treatment causes the synthesis of many stress-induced proteins with molecular weights of 70–72, 52, 37, 24, and 23 kDa in all genotypes. But in the tolerant genotype GE-415, the synthesis of a 54 kDa protein occurs under a NaCl treatment, which is not observed in the salt-sensitive genotype VL-481 (Uma et al., 1995). This suggests that the synthesis of NaCl-induced proteins in finger millet is correlated with the differences in the salt tolerance of the genotypes. In a species of citrus, either in cell suspension or when seedlings are grown in the presence of 0.2 M NaCl, the steady-state level of a citrus-LEA5 (C-LEA5) protein increases (Naot et al., 1995). Proteome analysis has allowed the identification of several novel proteins that are synthesized in plants in response to salinity (Tada and Kashimura, 2009; Yang et al., 2009). Yang and coworkers (2009) identified 38 salt-responsive proteins in the leaves of *Populus cathayana* Rehder by the peptide mass fingerprint (PMF) analysis. Some of the novel salt-responsive proteins identified were suggested to be involved in physiological and biochemical responses to salt stress in *P. cathayana*. A novel salt-responsive protein, not previously detected by the expressed sequence tag analysis or the transcriptome analysis, was identified in the mangrove plant *B. gymnorhiza* using comparative two-dimensional electrophoresis, which may provide insight into the salt-tolerance mechanism of the mangrove plant (Tada and Kashimura, 2009).

In many plant species, certain hydrophilic proteins and their mRNAs have been reported to be synthesized *de novo* in response to salt stress. Some of these proteins and mRNAs are also induced by a water deficit treatment with abscisic acid (ABA) (Artlip and Funkhouser, 1995). Such proteins have been grouped in different classes based on DNA sequences of their genes and/or predicted functions of the proteins (Bray, 1995). In most of the cases, the functions of the inducible protein have not been clearly established, and predicted functions have been proposed based on deduced amino acid sequences (Bray, 1995). Salinity imposition during the period of seed development following maturation leads to the synthesis of late embryogenesis-abundant (LEA) proteins in cotton, carrot, barley, and maize (Ramagopal, 1993). The LEA proteins contribute to salinity tolerance, desiccation tolerance, and freezing tolerance in plants by playing roles in ion sequestration, water replacement, and stabilization of macromolecules and membranes. The LEA proteins can act *in vitro* as glassy-state stabilizers, cryoprotectants, and molecular sponges; prevent protein aggregation; and bind to lipid vesicles or actin filaments (Figure 19.4). In vegetative organs of many other plant species, salinity-induced proteins that share significant amino acid sequences with the LEA proteins of cotton have been identified. Thus, various groups of LEA proteins have been identified, which have been classified based on notable structural domains predicted by amino acid sequences (Bray, 1995). LEA group 1 proteins include the Em family of proteins, which are devoid of cysteine and tryptophan. Such proteins have been identified in many monocots and dicots, and it is suggested that these proteins function in a water-binding capacity creating a protective aqueous environment (Bray, 1995). In salt-treated finger millet seedlings, a 21 kDa protein belonging to LEA group 1 of proteins appeared as the most prominent, whereas cucumber, a salinity-sensitive species, did not show any detectable level of expression of this protein when exposed to salt stress. The relatively tolerant species, such as finger millet and proso millet, showed higher levels of expression



**FIGURE 19.4** Roles of LEA proteins in freezing tolerance, salinity tolerance, and desiccation tolerance. Functions of LEA proteins in water replacement, in ion sequestration, and in protection of macromolecules and membranes have been proposed. LEA proteins can act *in vitro* as glassy-state stabilizers, cryoprotectants, and molecular sponges, and can prevent protein aggregation and bind to lipid vesicles or actin filaments.

compared to the sensitive species *setaria*, signifying the relevance of the 21 kDa protein in stress tolerance (Aarati et al., 2003). LEA group 2 proteins include dehydrin, RAB (ABA-responsive), and D11 proteins, which have characteristic lysine-rich regions and are also expressed owing to treatment with ABA (Bray, 1995). LEA group 3 and group 5 proteins are represented by D7 and D29 from cotton, respectively, and contain a repeated tract of 11 amino acids (Bray, 1995). Citrus cell suspensions grown in the presence of 0.2 M NaCl or leaves of citrus plants irrigated with NaCl accumulate a protein (C-LEA5) that has a high similarity with the cotton LEA5 protein (Naot et al., 1995). LEA group 4 protein represents the D113 protein, the synthesis of which is induced in drying cotton seeds, and this protein has a homologue in tomato LE25 (Bray, 1995). In addition to the inducible proteins that have predicted functions, there are many salt-induced proteins that have no specified functions. A protein, RD22, is induced early during seed development in *Arabidopsis* and has homology to an unidentified seed protein from *V. faba* (Bray, 1995). In the roots of salt-stressed barley plants, a “germin”-like protein has been identified (Bray, 1995). Germin is a protein that accumulates during the early growth of wheat plants and has no specified functions.

The most extensively studied proteins from many plant species that accumulate in response to dehydrative conditions like salinity, drought, and low temperature are dehydrins (group 2, LEA), which are hydrophilic Gly-rich proteins (Close, 1997). Although the specific biochemical function of dehydrins has not been demonstrated, they seemingly appear to play a role in protecting membranes from damage (Puhakainen et al., 2004) or act as surfactants that are capable of inhibiting the coagulation of a range of macromolecules and, thereby, preserving structural integrity (Close, 1997). The overexpression of wheat dehydrin *DHN-5* has been shown to enhance tolerance to salinity in *A. thaliana* (Brini et al., 2007).

Transgenic rice plants expressing a LEA protein gene (*HVA1*) from barley showed accumulation of the *HVA1* protein in both roots and leaves, and such plants showed increased tolerance to salinity (Xu et al., 1996). OsLEA3 is a LEA group 3 protein of rice (*O. sativa* L.), the expression of which is induced under salt stress. The accumulation of the OsLEA3 protein in the vegetative tissues of transgenic rice plants enhanced their tolerance to salt stress (Hu, 2008). These observations suggest that LEA proteins play an important role in the protection of plants under salt stress conditions, and that LEA genes hold considerable potential for use as molecular tools for genetic crop improvement toward salinity tolerance (Xu et al., 1996).

The proteomic approach applied to identify differentially expressed proteins in rice panicles in response to salt stress revealed that many proteins were involved in several salt-responsive mechanisms. Such proteins were involved in increasing plant adaptation to salt stress. Some of these proteins had a higher constitutive expression level, and some were associated with up-regulation of antioxidants and up-regulation of proteins involved in translation, transcription, signal transduction, ATP generation, etc. (Dooki et al., 2006). A subcellular proteomics approach was used to identify differentially expressed PM-associated proteins in the salt-tolerant rice variety IR651 in response to salinity. Most of the identified proteins were known to be involved in several important mechanisms of plant adaptation to salt stress, including proteins involved in the regulation of PM pumps and channels, membrane structure, oxidative stress defense, signal transduction, protein folding, the methyl cycle, etc. (Nohzadeh et al., 2007).

### 19.2.1.2 Protein Level in Salt-Stressed Plants

Protein synthesis in plants growing in saline environments is adversely affected. Salt stress results in an overall decrease in protein synthesis with a loss of polyribosomes (Artlip and Funkhouser, 1995). In germinating seeds, as well as during late growth stages of plants, salinity causes impairment in synthesis as well as degradation of proteins. To access the general impact of salt damage on plant growth and metabolism, various investigators have attempted to study the overall status of total proteins and soluble proteins and the pattern of protein synthesis in different parts of plants growing under salinity stress. Salinity in the majority of cases lowers the level of protein in salt-stressed plant parts as a result of the decreased synthesis of protein as well as due to the increased activities of protein-hydrolyzing enzymes. In certain cases, however, an increased protein level is noticed under salinization, possibly due to the increased synthesis of new salt-induced proteins or the decreased activities of proteolytic enzymes.

The process of protein synthesis has shown to be salt sensitive, as observed in wheat germ and *Suaeda maritima* (Greenway and Munns, 1980). Under *in vitro* conditions, a protein-synthesizing system extracted from these species is more sensitive to  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  than under *in vivo* conditions, as revealed by amino acid incorporation data. A salt-tolerant species like *S. maritima* can maintain optimum growth at a high salt level of 500 mM NaCl, whereas the enzymes extracted from this plant are sensitive to a low level of NaCl (170 mM). This suggests that under *in vivo* conditions, soluble enzymes as well as enzymes of protein-synthesizing mechanisms are comparatively less sensitive to NaCl than isolated enzymes under *in vitro* conditions. Although studying the effects of salt on protein synthesis in *S. maritima*, Hall and Flowers (1973) observed that in this species, protein synthesis is sensitive to salts and that amino acid incorporation decreases under *in vitro* conditions when the KCl concentration in the medium exceeds 50 mM.

In various crop species, a decrease in the protein level in salt-stressed plant parts is attributed to a decrease in protein synthesis, the decreased availability of amino acids, and the denaturation of the enzymes involved in amino acids and protein synthesis (Hall and Flowers, 1973; Popova et al., 1995). In chickpea (*Cicer arietinum* L.), one of the major legume crops for semiarid tropics, a salinity treatment with 100 mM NaCl in a nutrient solution caused a marked decrease in the level of proteins in developing seeds when plants were raised in sand cultures (Murumkar and Chavan, 1986).

When rice (*O. sativa* L.) seeds were germinated under increasing levels of NaCl salinity, a decrease in the total as well as the soluble protein level was observed in the embryoaxes (Dubey and Rani, 1987). A greater decrease in the protein level was observed in the embryoaxes of salt-sensitive cultivars than tolerant cultivars under a similar level of salinization. Dubey and Rani (1987) observed that under 14 dS  $\text{m}^{-1}$  NaCl salinity, the soluble protein level of embryoaxes of salt-sensitive rice cultivars Ratna and Jaya got reduced to almost one-third compared with nonsalinized seeds at 120 h of germination. A moderate salinity level of 7 dS  $\text{m}^{-1}$  NaCl had virtually no effect on the change in the total and soluble protein levels in embryoaxes of germinating seeds of salt-tolerant rice cultivars CSR-1 and CSR-3, whereas a higher salinity level caused a marked decrease in the protein level in the embryoaxes of these cultivars (Dubey and Rani, 1987). In barley plants,

the imposition of NaCl stress leads to a decrease in the leaf protein content and induces marked quantitative and qualitative changes in the polypeptide profiles, affecting mainly the proteins with approximately equal mobility (Popova et al., 1995).

Although salinity causes decreased protein synthesis and increased proteolysis in various plant species, in many cases increased protein levels are observed under salinization in germinating seeds (Dubey and Rani, 1987), growing seedlings (Dubey and Rani, 1989), and different plant parts (Elsamad and Shaddad, 1997). In germinating seeds, endospermic protein hydrolysis is suppressed under salinization. When seeds of rice cultivars differing in salt tolerance are germinated under increasing levels of NaCl salinity, it has been observed that salt treatment suppresses protein depletion from the endosperm of all cultivars, with greater suppression in salt-sensitive cultivars than in salt-tolerant cultivars (Dubey and Rani, 1987).

An apparent increase in the protein level in the endosperms of germinating seeds is observed with an increase in salinity. This can be explained as a result of decreased proteolysis caused by salinity leading to slower depletion of reserve proteins, and not as a result of enhanced protein synthesis (Dubey and Rani, 1987). The NaCl salinity caused a delay in the breakdown of endospermic proteins as well as inhibition in the translocation of hydrolyzed products from endosperms to growing embryoaxes (Dubey, 1983a). The obvious implication is that the inhibition of seedling growth under salinization can also be partly attributed to the delayed mobilization of reserve proteins, because proteolysis is probably the primary, but essential, step toward the synthesis of new proteins for seedlings growth (Ryan, 1973).

When raised under increasing levels of NaCl salinity, rice seedlings show an increased level of total as well as soluble proteins compared with nonstressed seedlings (Dubey and Rani, 1989). During a 5–20 day growth period, when two sets of rice cultivars differing in salt tolerance were examined for the metabolic status of proteins in roots and shoots, it was observed that under a salinity treatment level of  $14 \text{ dS m}^{-1}$  NaCl, the seedlings of tolerant cultivars maintained higher levels of total as well as soluble proteins compared to the seedlings of the sensitive cultivars (Dubey and Rani, 1989). The increased soluble protein level in rice seedlings became more evident when the salinity treatment was raised from a moderate level of  $7 \text{ dS m}^{-1}$  NaCl to a higher level of  $14 \text{ dS m}^{-1}$  NaCl (Dubey and Rani, 1989).

Similar to rice, the NaCl salinity treatment caused an increase in the protein content in cowpea (*Vigna unguiculata* L.) seedlings, pea (*Pisum sativum* L.), Trigonella (*Trigonella foenum-graecum* L. and *T. aphanoneura* Rech. f.), beans (*Phaseolus vulgaris* L.), and *Cajanus cajan* plants as well as in soybean callus cultures (Joshi, 1987; Mehta and Vora, 1987; Vyas and Rao, 1987; Elenany, 1997; Niknam et al., 2006; Dantas et al., 2007). The increased protein level under salinization as noted in these cases appears to be due to the increased synthesis of preexisting as well as certain new sets of proteins (Dubey and Rani, 1989). The increased synthesis of many novel proteins has been noticed in plants subjected to saline stress, but whether such increased synthesis is responsible for a net increase in the total and soluble protein levels of stressed plants remains to be investigated.

To understand the mechanism of salt tolerance in crops, various investigators have studied the metabolic status of proteins and amino acids in germinating seeds and seedlings using cultivars differing in salt tolerance (Dubey and Rani, 1987, 1989; Elsamad and Shaddad, 1997; Moons et al., 1997; Ahmed et al., 2001; Dantas et al., 2007). Especially in rice (*O. sativa* L.), a staple food crop for the majority of the world population, salt-tolerant cultivars are characterized by a higher value of protease-specific activity as well as a higher total and soluble protein content in germinating seed parts under control and salt treatments, when compared with sensitive seedlings (Dubey and Rani, 1989). This shows the salt-tolerance ability to be associated with a possible higher protein level in rice, seemingly endogenous proteins that are either not found or are very poorly expressed in sensitive cultivars.

Using a salt-tolerant rice variety, Pokkali, and a salt-sensitive variety, Taichung N1, a cDNA clone *oslea3*, encoding a LEA group 3 protein, was identified that accumulated to higher levels in the salt-tolerant variety compared with the sensitive one on imposition of salt stress (Moons et al., 1997). Further, this stress-induced protein and its mRNA declined less rapidly on sustained salt

shock in tolerant cultivars than the sensitive ones. Such observations indicate that the differential regulation of protein expression is associated with varietal differences in salt stress tolerance (Moons et al., 1997).

Soybean (*Glycine max*) cultivars differing in salt tolerance show different levels of proteins and amino acids when grown in the presence of NaCl (Elsamad and Shaddad, 1997). Salt-tolerant soybean cultivars, Clark and Forest, accumulate higher levels of soluble proteins, whereas the sensitive cultivar Kint shows a decrease in the soluble protein level when grown in saline soils (Elsamad and Shaddad, 1997). These observations indicate that in rice and soybean plants, the salt-tolerance ability is associated with a higher level of proteins, which are seemingly endogenous proteins that are either not found or are very poorly expressed in sensitive cultivars on imposition of salinity.

### 19.2.1.3 Enzyme Levels in Salt-Stressed Plants

Salinity induces changes in the activities of proteolytic (Dubey, 1983a; Dubey and Rani, 1987, 1990), amylolytic (Dubey, 1983a,b; Dreier et al., 1995), nucleolytic (Dubey, 1985), phosphorolytic (Dubey et al., 1987; Dubey and Sharma, 1989, 1990; Banuls et al., 1995; Lin et al., 1997), oxidative (Mittal and Dubey, 1995), antioxidative (Piqueras et al., 1996; Azevedo-Neto et al., 2006; Khosravinejad et al., 2008; Shafi et al., 2009), photosynthetic (Popova et al., 1995), and nitrogen assimilatory enzymes (Dubey, 1997; Debouba et al., 2007), in germinating seeds and in growing plants. Salinity causes either an increase or a decrease in the activity of enzymes, depending on the nature of the enzymes, the extent of stress, the plant parts studied, and the genotypes of plant species differing in salt tolerance.

In the endosperms of germinating rice seeds, salinity causes a decrease in the activities of hydrolytic enzymes  $\alpha$ -amylase, protease, ribonuclease, phosphatase, and phytase (Dubey, 1983a,b; Dubey, 1985; Dubey and Rani, 1987). The decrease is more in salt-sensitive than in salt-tolerant varieties. In growing seedlings of rice, salinity enhances the activities of nucleases (Dubey, 1985), proteases (Dubey, 1985; Dubey and Rani, 1990), peptidases (Dubey and Rani, 1990), phosphatases (Dubey et al., 1987; Dubey and Sharma, 1989, 1990), and oxidases (Mittal and Dubey, 1991). Genotypes of rice species differing in salt tolerance maintain different levels of salinity-induced activities of enzymes.

Barley plants grown in the presence of 200 mM NaCl show stimulation in  $\beta$ -amylase activity in leaves (Dreier et al., 1995).  $\beta$ -Amylases are regarded as stress-induced proteins in barley (Dreier et al., 1995). The NaCl treatment resulted in a decrease in the activities of  $\alpha$ -amylase,  $\beta$ -amylase, and  $\alpha$ -glucosidase enzymes involved in the seed germination process of two lentil cultivars, Castelluccio and Eston. In contrast, seeds of two bean cultivars, Pantelleria and Ustica, showed higher enzyme activities involved in the germination process, in response to salt stress (Sidari et al., 2008). Certain enzymes involved in the synthesis of osmolytes show a marked increase under salinity stress (Ramagopal, 1993). The enzymes of proline biosynthesis,  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS), and  $\Delta^1$ -pyrroline-5-carboxylate reductase; the penultimate enzyme of betaine biosynthesis, betaine aldehyde dehydrogenase, and the enzyme of sorbitol biosynthesis; aldose reductase, show increased activity in many plants subjected to salt stress (Ramagopal, 1993; Igarashi et al., 1997). Genes that code for key enzymes involved in osmolyte biosynthesis have been isolated from barley, spinach, sugar beet, soybean, and rice plants (Ramagopal, 1993). The salinity treatment of 7 and 14 dS m<sup>-1</sup> NaCl led to a significant increase in the levels of free amino acids with a substantial elevated level of proline in rice seedlings (Dubey and Rani, 1989). P5CS is the rate-limiting enzyme in proline biosynthesis in plants. It was observed that salt stress increased the P5CS activity, thus increasing the proline content in the roots of cucumber seedlings (Duan et al., 2006). Salt-tolerant varieties of rice show a higher level of expression of the enzyme P5CS and its mRNA compared with salt-sensitive varieties grown in the saline medium (Igarashi et al., 1997). Transgenic tobacco (*N. tabacum*) plants overexpressing the *p5cs* gene that encodes P5CS produced 10- to 18-fold more proline and exhibited better performance under salt stress (Kishor et al., 1995). Tobacco plants expressing *Arabidopsis* P5CS also showed higher levels of proline and enhanced resistance to the applied salt (Yamchi et al., 2007). The enzymes involved in membrane transport, such as PM-ATPase in cotton seedlings (Lin et al., 1997) and Ca<sup>2+</sup>-ATPase in tomato plants (Ramagopal, 1993), show a higher level of

activity under salinization. An increased turnover of the tonoplast  $H^+$ -ATPase was observed in the leaves of *C. sinensis* plants under salinity stress (Banuls et al., 1995).

The activities of the oxidative enzymes polyphenol oxidase and indole-3-acetic acid oxidase increase in the seedlings of salt-tolerant as well as salt-sensitive rice cultivars under salinization, and the extent of the increase differs in the two sets of cultivars (Mittal and Dubey, 1995). Similarly, phosphohydrolases show varying behavior under salinity in rice plants of differing salt tolerance.

The changes in the activity behavior of the enzymes acid phosphatase, alkaline phosphatase, and ATPase isolated from the chloroplasts of two sets of rice seedlings differing in salt tolerance when grown under increasing levels of NaCl salinity are shown in Table 19.2. As it is evident from the table, the acid phosphatase activity was more inhibited owing to salinity in salt-sensitive cultivars compared with the salt-tolerant ones, whereas the activity of alkaline phosphatase increased in the salt-sensitive seedlings but not in the salt-tolerant ones. Further, salinity caused an enhancement of the ATPase activity in both sets of rice seedlings, with a greater enhancement in tolerant cultivars than in the sensitive ones. The activities of the antioxidant enzymes superoxide dismutase (SOD), peroxidase, and glutathione reductase (GR) increase in callus cultures of *Citrus limon* (Piqueras et al., 1996), *O. sativa* (Elenany, 1997), and *Medicago sativa* (Safarnejad et al., 1996) grown in the presence of NaCl. In soybean callus cultures, an increase in the GR activity in response to NaCl constitutes an adaptive response of callus tissues to NaCl (Elenany, 1997).

The level and activity of the key enzyme of photosynthesis in  $C_3$  plants, ribulose-1,5-bisphosphate carboxylase, decrease in barley plants on the imposition of NaCl stress (Popova et al., 1995). The prime enzyme of nitrate assimilation, nitrate reductase (NR), has been extensively studied for its behavior in different plant species under salinization (Dubey and Pessarakli, 1995; Dubey, 1997; Debouba et al., 2007). Salinity effects on NR activity are varied and depend on the type and extent of salinity as well as on the genotypes of the plants studied. In intact tissues of wheat, lentil (*Lens esculenta* Moench), mulberry (*Morus abla*), sorghum, and tobacco plants, the NR activity decreases owing

**TABLE 19.2**  
**Salinity-Induced Alteration in the Activity Behavior**  
**of Phosphorolytic Enzymes in Chloroplasts**  
**of 20-Day-Grown Rice Plants**

| Rice Cultivars | NaCl Treatment (dS m <sup>-1</sup> ) | Acid Phosphatase | Alkaline Phosphatase | ATPase |
|----------------|--------------------------------------|------------------|----------------------|--------|
| CSR-1 (T)      | 0                                    | 4.00             | 0.68                 | 0.12   |
|                | 7                                    | 3.20             | 0.50                 | 0.15   |
|                | 14                                   | 3.00             | 0.45                 | 0.22   |
| CSR-3 (T)      | 0                                    | 4.30             | 0.70                 | 0.12   |
|                | 7                                    | 3.20             | 0.64                 | 0.16   |
|                | 14                                   | 3.00             | 0.48                 | 0.26   |
| Ratna (S)      | 0                                    | 3.20             | 0.62                 | 0.06   |
|                | 7                                    | 2.60             | 0.70                 | 0.08   |
|                | 14                                   | 1.40             | 0.76                 | 0.11   |
| Jaya (S)       | 0                                    | 2.80             | 0.59                 | 0.05   |
|                | 7                                    | 2.00             | 0.64                 | 0.08   |
|                | 14                                   | 1.20             | 0.68                 | 0.14   |

*Notes:* T and S in parentheses indicate tolerant and sensitive rice cultivars, respectively. Enzyme units are expressed as  $\mu\text{mol}$  substrate hydrolyzed  $\text{h}^{-1} \text{mg}^{-1}$  protein.



to NaCl salinity (Dubey, 1997), whereas, in rice plants, the behavior of NR varies in genotypes differing in salt tolerance when raised under NaCl salinity (Katiyar and Dubey, 1992). The salt-sensitive rice cultivars Ratna and Jaya show a decrease in the shoot and root, whereas the salt-tolerant cultivars CSR-1 and CSR-3 show increased activity of the enzymes in both of these tissues under salinization (Katiyar and Dubey, 1992).

Isoenzyme profiles of many enzymes are influenced by salinity. In certain cases, some of the molecular forms of enzymes present in nonsalinized plants disappear in stressed plants, whereas, in other cases, certain new molecular forms of enzymes appear under salinization. In shoots of 15-day-old nonsalinized rice seedlings, four acid phosphatase isozymes were observed, whereas when seedlings were raised at a salinity level of 14 dS m<sup>-1</sup> NaCl, only one isoenzyme remained detectable (Dubey and Sharma, 1989). The decreased number of acid phosphatase isoenzymes at a higher level of salinization paralleled the decreased activity of the enzyme under such conditions (Dubey and Sharma, 1989).

In the young embryoaxes of germinating seeds, certain new molecular forms of acid phosphatases appear under salinization. When acid phosphatase isoforms from the embryoaxes of germinating seeds of the salt-sensitive rice cultivar Jaya and the salt-tolerant cultivar CSR-1 were compared at 48 and 96 h of germination under increasing levels of NaCl salinity, it was observed that certain new isoenzyme forms appeared in both sets of cultivars under salinization (Dubey and Sharma, 1990). Further, a great number of isoenzymes were observed in the embryoaxes of salt-tolerant rice varieties than in those of salt-sensitive varieties under both controls as well as salt treatments (Dubey and Sharma, 1990). Therefore, under salinization, certain isoforms of acid phosphatase are not synthesized, whereas the synthesis of certain new isoforms is induced depending on the plant parts and the genotypes studies. It has been shown that salt tolerance is associated with the presence of a large number of acid phosphatase isoenzymes (Dubey and Sharma, 1990).

The specific activities and patterns of peroxidase and SOD isoenzymes are altered significantly in plants subjected to salinity stress. Rice seedlings differing in salt tolerance possess constitutively different number of peroxidase isoforms in nonsalinized seedlings. When these seedlings were raised under NaCl salinity, certain new isoforms of peroxidases appeared, and the intensities of some of the preexisting isoenzymes increased. In 15-day-old seedlings of a salt-tolerant rice cv. CSR-1, three isoenzymes were observed in roots and five in shoots, whereas in a salt-sensitive cv. Ratna, six isoenzymes were observed in the roots as well as in the shoots (Mittal and Dubey, 1991). Salt-tolerant embryonic callus cultures of lemon (*C. limon* L. Burn) exhibited an increase in the activity of antioxidant enzymes involved in oxygen metabolism with an increase of peroxidase activity and with the induction of a new SOD isozyme (Piqueras et al., 1996). These studies and other similar studies suggest that peroxidase and SOD isoenzymes can serve as useful markers in the analysis of gene functions and metabolic regulations, including salt-tolerance characteristics (Mittal and Dubey, 1991).

Like acid phosphatase and peroxidases, isoenzyme profiles of ribonucleases (Mittal and Dubey, 1990) and  $\alpha$ -amylases (Dubey, 1983b) are also influenced under salinization. The presence of different isoenzyme patterns of phosphatases, peroxidases, SOD, and ribonucleases in salt-sensitive and salt-tolerant genotypes of crops strengthens the view that salt tolerance or sensitivity depends on the genetic and biochemical makeup of the species. Also, specific molecular forms of the isoenzymic proteins, which appear to be constitutive proteins, are possibly associated with the salt-tolerance or sensitivity characteristics. However, the mechanisms of the expression of intrinsic isoenzyme proteins related with sensitivity or tolerance and of those isoenzymic proteins that specifically appear under salinization remain to be investigated.

### 19.2.2 DROUGHT

Water is the earth's most distinctive constituent and is an essential ingredient of all life. Its deficit is one of the most common environmental factors limiting crop productivity. Drought is a natural calamity and has devastating effects on crop yields. Crop plants are frequently subjected to drought

during the course of their lifetimes. However, certain stages, such as germination, seedling, and flowering, are the most critical for water deficit damage. Stress imposed during these stages drastically affects crop yields. Drought reduces plant growth and manifests several morphological and biochemical alterations in plants, ultimately leading to a massive loss in yield. A reduction in the efficiency of key processes including protein synthesis, photosynthesis, respiration, and nucleic acid synthesis is among the biochemical manifestations of drought. Drought inhibits protein synthesis, induces the synthesis of small sets of stress-specific proteins, promotes important modification in gene expression, causes activation or inhibition in the activities of many enzymes, and leads to changes in the ultrastructures of tissues. A considerable amount of work has been done by various groups of investigators in the last few years to understand the mode of protein synthesis in plant parts under drought conditions (Bewley and Larsen, 1982; Dasgupta and Bewley, 1984; Heikkilä et al., 1984; Baker et al., 1995; Zhang et al., 1996; Close, 1997; Pelah et al., 1997; Bibi et al., 2009; Ke et al., 2009), the level of proteins in stressed plants (Rai et al., 1983; Kumar and Singh, 1991), and the activities of key enzymes influenced under drought (Lodh et al., 1977; Mali et al., 1980; Geigenberger et al., 1997; Igarashi et al., 1997; Sharma and Dubey, 2004, 2005a,b).

#### 19.2.2.1 Drought-Induced Protein Synthesis

Drought causes alteration in the gene expression in plants, leading to an inhibition of protein synthesis as well as an enhanced synthesis of certain stress-specific proteins. Quantitative and qualitative changes occur in the synthesis of proteins in plants in response to water deficit. It is well documented that in various crops, drought causes a tissue- and organ-specific differential genomic expression, which results in changes in the patterns of protein synthesis in cells (Bewley and Larsen, 1982; Dasgupta and Bewley, 1984; Heikkilä et al., 1984; Yoshimura et al., 2008; Ke et al., 2009; Si et al., 2009). Plants growing in drought condition or cells undergoing adaptation to drought show both a decrease as well as an increase in small sets of cellular proteins. Many of these proteins that are specifically synthesized under drought have been isolated and well characterized (Singh et al., 1987; Claes et al., 1990; Close, 1997; Pelah et al., 1997). Evidence is increasing in favor of a relationship between the accumulation of drought-induced proteins and physiological adaptations to water limitation (Riccardi et al., 1998).

A mild to moderate drought condition decreases the efficiency of protein synthesis in plants, but such plants recover and their protein synthesis returns to normal when water stress is reversed or plants are rewatered (Bewley and Larsen, 1982). The imposition of drought alters the status of the protein-synthesizing complex polyribosomes in the tissues. The content of polyribosomes decreases with one set of drought stress, and the extent of such a decrease varies among different plant species and even in different organs of the same plant (Scott et al., 1979; Bewley and Larsen, 1982). Studies in maize (Bewley and Larsen, 1982) and wheat (Scott et al., 1979) have indicated that increasing the level of drought causes a decrease in the polyribosome level. Plant species that can survive under drought stress show a greater capacity to produce polyribosomes in the tissues.

Various investigators have demonstrated the synthesis of drought-specific proteins in different crops (Dasgupta and Bewley, 1984; Heikkilä et al., 1984; Singh et al., 1987; Claes et al., 1990; Baker et al., 1995; Zhang et al., 1996; Close, 1997; Pelah et al., 1997). Many of these proteins also appear in response to the application of ABA, suggesting that ABA is a signal in the stress response. Genes encoding these proteins have been isolated and studied using DNA probes. Like salinity stress, drought-inducible proteins have been grouped in several families depending on the DNA sequences of genes, their expression characteristics, and their predicted functions.

The induced synthesis of novel proteins, including enzymes involved in the biosynthesis of osmoprotectants, HSPs, LEA proteins, chaperones, enzymes associated with detoxification, transcription factors, kinases, and phosphatases, in response to drought has been reported in several plant species (Jiang and Huang, 2002; Khurana et al., 2008; Ke et al., 2009). Major families of drought-induced proteins have been described as LEAs, RABs, dehydrins, and vegetative storage proteins (Artlip and Funkhouser, 1995). LEA proteins have been further subdivided into several groups: group 1

(D19 protein from cotton), group 2 (D11 from cotton), group 3 (D7 from cotton), and group 5 (D29 from cotton). Many of these proteins are hydrophilic and are soluble on boiling, and are therefore expected to be located in the cytosol. The precise function of LEAs is still unknown, but substantial evidence indicates their involvement in desiccation tolerance (Khurana et al., 2008). It is predicted that most of these proteins are involved in protecting cellular structures and components from dehydration associated with water deficit.

Among proteins in plants that accumulate in response to dehydrative forces or drought, dehydrins have been the most commonly observed. Rice, barley, maize, pea, and *Arabidopsis* plants show an increased synthesis of dehydrins under osmotic stress (Ramagopal, 1993). Dehydrins are composed of several typical domains joined together in a few characteristic patterns with numerous minor permutations. Dehydrin polypeptides are made up of less than 100 to nearly 600 amino acid residues (Close, 1997). Although the fundamental biochemical mode of action of dehydrins has not been demonstrated, it is believed that dehydrins are surfactants and, thereby, they inhibit the coagulation of a range of macromolecules and preserve the structural integrity of the cell (Close, 1997). Genes encoding dehydrins are also ABA regulated. In dehydrated leaves of tomato, maize, and *Arabidopsis* plants, endogenous ABA levels increase with the simultaneous increase in the levels of dehydrins and their mRNAs (Bray, 1995). Dehydrins are localized primarily in the cytoplasm of root and shoot cells (Bray, 1995).

In certain plant species, the synthesis of dehydrin-like proteins has been observed under osmotic stress or under treatment with ABA. In *Stellaria longipes*, the synthesis of a dehydrin-like protein is induced as a result of treatment with ABA or under osmotic stress (Zhang et al., 1996). A sequence analysis of this protein indicates that it shares some similarity in structural features with dehydrins of other plants and also exhibits certain unique characteristics (Zhang et al., 1996). In castor bean, the synthesis of dehydrin-like proteins is tissue specific and is dependent on the physiological stage of the seed. Patterns of water-deficit-induced dehydrin-related polypeptides in endosperms differ from those induced during late seed development (Han and Kermode, 1996). In drought-stressed roots and shoots of *Lathyrus sativus*, dehydrin-like transcripts accumulate, which are also expressed in unstressed seedlings owing to the ABA treatment (Sinha et al., 1996). A novel protein with 40 kDa molecular weight has been detected in pea plants under desiccation. The deduced amino acid sequence of this protein indicates two lysine-rich blocks; however, the remainder of the sequence differs markedly from other pea dehydrins (Robertson and Chandler, 1994). By analogy with heat-shock cognate proteins, this protein has been designated as a dehydrin cognate (Robertson and Chandler, 1994). Transgenic *Arabidopsis* plants overexpressing the wheat dehydrin *DHN-5* were shown to be more tolerant to water deprivation as compared to wild-type plants (Brini et al., 2007). It was proposed that Dhn-5 contributes to an improved tolerance to drought stress through osmotic adjustment (Brini et al., 2007).

Like dehydrins, the RAB and D11 (group 2) family of proteins are also ABA regulated and possess a characteristic lysine-rich region with consensus amino acid sequences repeated at least two times. Proteins of this family have been identified in many plant species, including maize, tomato, wheat, alfalfa, *Arabidopsis*, rice, and castor (Mundy and Chua, 1988; Ramagopal, 1993; Bray, 1995; Han and Kermode, 1996). LEA group 1 (D19) proteins, which are devoid of cysteine and tryptophane, have been detected in cotton, barley, and carrot under water deficit (Ramagopal, 1993). It is suggested that LEA group 1 proteins function in a water-binding capacity creating a protective aqueous environment (Bray, 1995). LEA group 3 and LEA group 5 proteins, which are represented by D7 and D29 proteins, respectively, from cotton contain a repeated tract of 11 amino acids. These proteins have been isolated from desiccating mature cotton embryos, chloroplasts of *Craterostigma plantagineum*, and citrus seedlings exposed to drought (Bray, 1995; Naot et al., 1995). A citrus cell suspension in response to salt stress, leaves of citrus plants irrigated with NaCl, or seedling exposed to drought led to an osmotic stress-induced elevated level of LEA5 proteins and their mRNAs (Naot et al., 1995). Another group of proteins, group 4, is represented by the D113 protein, which has a homologue in tomato and is expressed in drying cotton seeds (Ramagopal, 1993; Bray, 1995).

Using a transgenic approach, it is suggested that LEA proteins play an important role in the protection of plants under drought stress. The expression of the barley (*Hordeum vulgare* L.) LEA protein gene, *HVA1*, in rice cell suspension leads to a higher level of accumulation of this protein, and such plants show an increased tolerance to water deficit (Xu et al., 1996). LEA group 4 genes from the resurrection plant *Boea hygrometrica* were shown to confer dehydration tolerance in transgenic tobacco (Liu et al., 2009). These proteins are likely to play a role in the general protection of the plant cells during dehydration and affect membrane and protein stability. LEA4-1 cDNA cloned from *Brassica napus* was overexpressed in transgenic *Arabidopsis* plants. The transgenic *Arabidopsis* plants expressing the *BnLEA4-1* gene under either the constitutive CaMV35S or the abiotic stress-inducible RD29A promoter showed an enhanced tolerance to drought (Dalal et al., 2009).

Osmotin, the 26 kDa protein, which is synthesized and accumulates in cells undergoing osmotic adjustment to NaCl, also accumulates in cells undergoing osmotic adjustment to PEG (Singh et al., 1987). ABA, which is known to induce osmotic adjustment in cells, also induces the synthesis of osmotin. Osmotin synthesis is regulated by ABA, but its accumulation is dependent on the extent of drought stress and the adjustability of the cells to stress. Like dehydrins, osmotin also is the much extensively studied protein that accumulates under water and salinity stresses in several plant species like tobacco, triplex, tomato, and maize (Ramagopal, 1993). An osmotically regulated glycine- and threonine-rich protein was identified in rice by Mundy and Chua (1988). This protein is a product of a small ABA-responsive gene, *rab 21*. Osmotic stress imposed by PEG or dessication leads to an increase in the level of ABA, and, in turn, the *rab 21* gene is induced and expressed to synthesize these proteins in rice tissues. The constitutive expression of an osmotin gene in transgenic tobacco was shown to improve its tolerance to drought stress (Barthakur et al., 2001). Parkhi and coworkers (2009) provided a direct evidence for a protective role of osmotin in cotton plants experiencing drought stress by transforming cotton plants with apoplastically secreted tobacco osmotin. Under PEG-mediated drought stress, the osmotin-expressing seedlings showed better growth performance, and higher relative water content and proline levels, while showing reduced H<sub>2</sub>O<sub>2</sub> levels, lipid peroxidation, and electrolyte leakage.

From rice cv. Tainchung native 1, a 15 kDa protein that accumulates in the sheaths and roots of mature plants and seedlings when subjected to either osmotic stress or after treatment with ABA has been isolated and characterized (Claes et al., 1990). Rice varieties show considerable differences in sensitivity to drought; however, in many of the varieties examined, water deficit created as a result of PEG led the induced synthesis of one 26 kDa protein with a pI of 6 (Perezmolphebaltch et al., 1996). In certain varieties of rice, drought causes the accumulation of 18 and 85 kDa proteins, called stress-associated proteins, that also accumulate under salinity and high and low temperatures (Pareek et al., 1997).

A boiling-stable protein (BspA) has been shown to accumulate in the roots of *Populus popularis* plants under drought (Pelah et al., 1997). In addition to BspA, plants also show accumulation of the drought stress-related proteins dehydrin (dsp-16) and sucrose synthase (SS) under water deficit (Pelah et al., 1997). In a highly drought-tolerant legume, cowpea (*Vigna unguiculata*), which shows an about 160 times higher accumulation of ABA in drought-stressed conditions compared with unstressed plants, two cDNA clones were identified in drought-stressed plants, CPRD 8 and CPRD 22, which encode putative proteins that are related to old yellow enzyme and LEA group 2 proteins, respectively (Iuchi et al., 1996b). However, in cowpea plants dehydrated for 10h, two additional cDNA clones, CPRD 12 and CPRD 46, were identified, which encode putative proteins related to nonmetallo-short-chain alcohol dehydrogenase (ADH) (CPRD 12) and chloroplastic lipoxxygenase (CPRD 46). These genes are also induced under salinity stress (Iuchi et al., 1996a).

Exposure of *A. thaliana* to drought stress results in the accumulation of the RAB 18 protein, and such plants develop an enhanced freezing tolerance (Mantyla et al., 1995). A progressive water deficit in whole *Solanum tuberosum* plants leads to an about 2.5-fold increase in the leaf ABA content and the synthesis of two chloroplastic proteins of 32 and 34 kDa, namely, CDSP 32 and CDSP 34, which

are synthesized in the stroma and in the thylakoids, respectively (Pruvot et al., 1996). A 65 kDa protein with a *pI* value of 5.2 has been shown to accumulate gradually in tomato leaves during drought stress (Tabaeizadeh et al., 1995). The quantification of this protein by gold labeling indicates that the synthesis of this protein occurs in nuclei and chloroplasts as well as in some cytoplasmic regions of the cells in drought-stressed plants (Tabaeizadeh et al., 1995).

Certain additional families of proteins and their genes that are induced by water deficit have been identified in specific plant species. In *Arabidopsis*, 77.9 and 64.5 kDa hydrophilic proteins accumulate under drought stress, and their genes, which are adjacent to each other in the genome, have been characterized from different laboratories (Bray, 1995). The synthesis of these proteins also occurs with the application of ABA. Similarly, a family of genes and their products, glycine-rich proteins, which are hydrophilic and ABA responsive, have been identified in alfalfa plants under drought stress (Bray, 1995). However, no specific predictions about the functions of these *Arabidopsis* and alfalfa proteins have been made under stressful conditions.

A gene named *ATHB-7*, which belongs to a class of recently discovered homeobox genes found as yet only in plants, has been characterized in all organs of *A. thaliana*. The expression of this gene and the synthesis of its proteins, called homodomain-leucine zipper (HD-Zip) proteins, are induced severalfold under water deficit as well as by an exogenous treatment with ABA (Soderman et al., 1996). It is suggested that *ATHB-7* is transcriptionally regulated in an ABA-dependent manner and may act in a signal transduction pathway mediating a drought response (Soderman et al., 1996). Besides, cytosolic and organellar proteins, which are induced under drought stress, in the cell walls of bean (*P. vulgaris*) seedlings, two proteins, of 36 and 33 kDa, have been identified that are glycosylated and accumulate when plants are subjected to a gradual loss of water (Covarrubias et al., 1995).

HSPs are known to accumulate in plants under drought stress (Arora et al., 1998; Jiang and Huang, 2002). One of the presumed functions of HSPs is related to the prevention of protein denaturation during cellular dehydration. The overexpression of *AtHsp90.2*, *AtHsp90.5*, and *AtHsp90.7* in *A. thaliana* enhanced plant sensitivity to drought stress (Song et al., 2009). When a rice small HSP gene, *sHSP17.7*, was overexpressed in the rice cultivar Hoshinoyume, an enhanced tolerance to drought stress was observed in transgenic lines (Sato and Yokoya, 2008). At the end of drought treatments, only transgenic seedlings with higher expression levels of the *sHSP17.7* protein were found to regrow after rewatering. Transgenic tobacco plants constitutively expressing elevated levels of NtHSP70–1 showed an enhanced tolerance to drought stress (Cho and Choi, 2009).

Studies conducted by various investigators related to the effects of drought stress on the protein synthesis in many important crops suggest that drought stress severely affects protein synthesis, alters gene expression and protein profiles in stressed tissues, and induces the synthesis of specific-stress-induced proteins. Many of these proteins are hydrophilic and belong to specified families and have predicted functions in protecting the cells from drought stress. Some of the stress-induced proteins appear to be tissue specific, whereas others do not appear to be specific for any particular tissue or organ. Genetic expression studies reveal that among the stress-induced proteins that are well characterized, the majority are the product of ABA-responsive genes. How stress conditions signal an increased production of ABA, how ABA modulates the expression of these genes, and what is the functional role of stress-responsive proteins in dehydration tolerance, such as osmoprotectants, radical scavengers, protectants of subcellular organelles and macromolecules, or as regulatory proteins, remain yet to be investigated in detail. An immunoblot analysis performed in the leaves of drought-tolerant (Katya and Zlatitza) and drought-sensitive (Sadovo and Miziya) wheat varieties for the detection of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), RUBISCO activase (RA), RUBISCO binding protein (RBP), dehydrins, and some HSPs and ATP-dependent calpain protease (Clp) proteins under drought stress revealed that the drought-tolerant varieties Katya and Zlatitza had higher levels of these proteins, especially RBP and Clp proteases, compared to the sensitive varieties (Demirevska et al., 2008).

### 19.2.2.2 Protein Level in Drought-Stressed Plants

The levels of total as well as soluble proteins get altered in plants growing under drought conditions compared with plants growing under nonstressed conditions. Various workers have observed either a decrease (Barnett and Naylor, 1966; Hsiao, 1973; Kumar and Singh, 1991; Gogorcena et al., 1995; Yu et al., 1996; Boo and Jung, 1999; Sharma and Dubey, 2005a; Bai et al., 2006) or an increase (Rai et al., 1983; Kumar and Singh, 1991) in the levels of total or soluble proteins in different organs of plants subjected to drought stress. The increased or decreased levels of proteins depend on the plant species and organ studied as well as the severity of the stress.

Sharma and Dubey (2005a) observed a concomitant decrease in the content of the total soluble protein with an increasing level of water deficit in roots as well as in shoots of growing rice seedlings. Shah and Loomis (1965) observed decreased contents of soluble and total proteins in sugar beet leaves from data recorded on a per gram of dry weight basis when the plants were subjected to progressive drought stress. These investigators observed that the response to drought was quick and could be reversed by rewatering the plants. This indicates that the effects of drought are reversible to a certain extent. According to Hsiao (1973), the rapid response of plants under drought stress and its quick reversibility by rewatering suggest that drought affects protein synthesis mainly at the translation level. When Bermuda grass plants were subjected to increasing levels of drought, a decrease in the soluble protein level was observed (Barnett and Naylor, 1966). In whole chloroplasts as well as in chloroplast membrane fractions isolated from drought-resistant as well as drought-sensitive genotypes of drought-stressed wheat plants, a decrease in the protein content was observed compared with nonstressed plants (Kulshrestha et al., 1987). Mung bean seedlings differing in drought stress tolerance, when raised under increasing water deficits, showed a decrease in the protein level in the axis (Kumar and Singh, 1991).

When seedlings of chickpea were used to investigate drought stress-induced variations in protein profiles of germinating cotyledons using the SDS-PAGE analysis, a quantitative decrease in the level of some high-molecular-weight proteins was observed on the fifth day of germination and some new germination-related proteins appeared on the seventh day in control. In cv. CM-2000, a delayed expression of two proteins with mol. wt. 100 and 60.8 kDa, respectively, was observed under drought stress, while these proteins were expressed earlier in cv. CM-94/99 (Bibi et al., 2009). In *Lycopersicon chilense* whole plants as well as in cell suspensions, drought stress leads to a decreased synthesis of a proline-rich 12.6 kDa protein in the cell walls (Yu et al., 1996). This is possibly attributed to the downregulation of its gene, designated PTGRP under desiccation. In the nodules of drought-stressed (−2.03 MPa) pea (*P. sativum* L. cv. Frilene) plants, an about 30% decline in the soluble protein level was observed compared with well-watered plants (Gogorcena et al., 1995). When rice varieties differing in drought stress tolerance were examined for changes in the protein profiles in different organs due to drought stress, it was observed that in the two cultivars, Sinaloa and IR 10120, the synthesis of several polypeptides decreased owing to PEG-induced drought (Perezmolphebalch et al., 1996). It is suggested that in rice, the extent of the decrease in the levels of proteins or changes in protein profiles in different organs due to water deficit are cultivar specific (Perezmolphebalch et al., 1996).

A decreased level of the total as well as of the soluble proteins in drought-stressed tissues (Kumar and Singh, 1991) appears to be due to more degradation of proteins as well as due to the overall inhibition in protein synthesis under drought. It has been observed that drought-stressed plant parts show a high protease activity compared with nonstressed plants (Thakur and Thakur, 1987). The high activity of protease in drought-stressed plants appears to be of adaptive significance, because it leads to the accumulation of free amino acids as a result of the degradation of proteins. Increased levels of free amino acids together with organic acids and quaternary ammonium compounds serve as compatible cytoplasmic solutes to maintain the osmotic balance between the cytoplasm and the vacuole under conditions of drought stress (Barnett and Naylor, 1966).

Genotypes of crop cultivars differing in drought tolerance, when raised under increasing levels of drought, show different levels of proteins as well as specific activities of protease. Seedlings of drought-tolerant mung bean genotypes show a higher protein content in embryoaxes as well as in cotyledons compared with drought-sensitive genotypes when raised at a  $-10.0$  bar moisture stress level (Kumar and Singh, 1991). Similarly, drought-resistant maize (*Zea mays* L.) cultivars show a high protease activity at higher levels of drought stress, whereas an inhibition in protease activity is noticed under higher drought stress levels in sensitive cultivars (Thakur and Thakur, 1987). While comparing the total protein and free amino acid pool size in drought-resistant and drought-sensitive cultivars of *C. arietinum* and *Z. mays*, Rai et al. (1983) observed that resistant plants are characterized by an increase over nonstressed plants in total protein and free amino acid levels.

Certain investigators have observed an increase in protein levels in plants subjected to drought stress (Rai et al., 1983; Kumar and Singh, 1991). Genotypes of *C. arietinum* cultivars, differing in drought stress tolerance, when raised under increasing osmotic potential levels, show increased protein levels in shoots compared with nonstressed plants (Rai et al., 1983). A drought-resistant *C. arietinum* cv. C-214 showed an increase of 60% protein over control at an osmotic potential of  $-3$  atm, whereas a sensitive cultivar, G-130, showed a 15% increase over control under similar conditions of stress (Rai et al., 1983). Similarly, when drought-resistant *Z. mays* cv. Ageti-76 plants were grown under increasing osmotic potentials in the range of 1–10 atm, an increase in the protein content was noticed, reaching 190% of control (Rai et al., 1983). Similarly, in the cotyledons of germinating mung beans under drought stress, an increased protein level was noticed when compared with nonstressed germinating seeds (Kumar and Singh, 1991). These observations indicate that drought has varying effects on the level of proteins in different crop species, and the stress-induced response depends on the species of the crop examined, and it may vary even in different organs within the same species.

### 19.2.2.3 Enzyme Levels in Drought-Stressed Plants

The normal metabolism of plants growing under drought conditions is adversely affected with a concomitant disturbance of the enzymatic constitution of the plants. Drought stress lowers the levels of many enzymes in the tissues (Mali et al., 1980; Thakur and Thakur, 1987; Gogorcena et al., 1995; Du et al., 1996; Reddy, 1996; Geigenberger et al., 1997; Sharma and Dubey, 2005b; Xu and Zhou, 2005). The activities of certain enzymes increase as a result of drought stress (Hsiao, 1973; Lodh et al., 1977; Thakur and Thakur, 1987; Bray, 1995; Igarashi et al., 1997; Sharma and Dubey, 2005a). Nitrogen assimilation and photosynthetic efficiency are reduced in drought-stressed plants mainly owing to the decreased activities of the key enzymes involved in these processes. NR, the prime enzyme in the N assimilation process, is markedly inhibited by drought (Hsiao, 1973; Xu and Zhou, 2005). The effect of mild ( $-0.5$  MPa) as well as moderate ( $-2$  MPa) levels of drought imposed for 24 h, on the level of protein, and the activities of enzymes NR, glutamine synthetase (GS), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) in the roots and shoots of 20-day-grown rice plants are shown in Table 19.3. As it is evident from the table, drought stress causes a drastic decline in the protein levels as well as in the activities of the enzymes of  $\text{NO}_3^-$  assimilation, NR and GS, whereas the key enzymes of amino acid metabolism, AlaAT and AspAT, show increased activity under a mild level of drought; however, under a moderate drought stress of  $-2$  MPa, a pronounced inhibition in the enzyme activity is noticed. The activity of NR is directly associated with protein synthesis and plant growth, and both of these processes are adversely affected under drought (Sinha and Nicholas, 1981). Rice seedlings subjected to severe drought stress showed a marked decline in the levels of both NRact (NR activity in the presence of  $\text{Mg}^{2+}$  representing the non-phosphorylated NR state) and NRmax (NR activity in the presence of EDTA representing maximum NR activity), whereas the NR activation state remained almost unaltered (Sharma and Dubey, 2005b).

**TABLE 19.3**  
**Drought Stress–Induced Decrease in the Level of Protein and Alteration in the Activity of Enzymes of N Metabolism in Roots (R) and Shoots (S) of 20-Day-Grown Rice Plants. Two Rice Cultivars were Used for the Study**

| Rice Cultivars | Drought Stress |     | Protein (mg g <sup>-1</sup> fw) | Nitrate Reductase                                                 | Glutamine Synthetase                                                     | Alanine Aminotransferase                                   | Aspartate                                                                       |
|----------------|----------------|-----|---------------------------------|-------------------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------|---------------------------------------------------------------------------------|
|                |                |     |                                 | (nmol NO <sub>2</sub> min <sup>-1</sup> mg <sup>-1</sup> Protein) | (μmol-γ-Glutamyl Hydroxamate min <sup>-1</sup> mg <sup>-1</sup> Protein) | (nmol Pyruvate min <sup>-1</sup> mg <sup>-1</sup> Protein) | Aminotransferase (nmol Oxaloacetate min <sup>-1</sup> mg <sup>-1</sup> Protein) |
| Ratna          | 0              | (R) | 13.00                           | 7.80                                                              | 0.56                                                                     | 18.00                                                      | 42.00                                                                           |
|                |                | (S) | 24.00                           | 8.50                                                              | 0.36                                                                     | 120.00                                                     | 30.00                                                                           |
|                | 0.5 MPa        | (R) | 6.00                            | 6.00                                                              | 0.48                                                                     | 32.00                                                      | 54.00                                                                           |
|                |                | (S) | 18.00                           | 7.50                                                              | 0.20                                                                     | 141.00                                                     | 67.50                                                                           |
|                | –2.0 MPa       | (R) | 2.50                            | 1.80                                                              | 0.36                                                                     | 7.00                                                       | 18.00                                                                           |
|                |                | (S) | 10.80                           | 2.00                                                              | 0.08                                                                     | 15.00                                                      | 17.50                                                                           |
| Jaya           | 0              | (R) | 6.00                            | 9.80                                                              | 0.82                                                                     | 30.00                                                      | 38.20                                                                           |
|                |                | (S) | 14.60                           | 11.0                                                              | 0.65                                                                     | 62.00                                                      | 45.00                                                                           |
|                | –0.5 MPa       | (R) | 5.00                            | 7.90                                                              | 0.75                                                                     | 42.50                                                      | 62.00                                                                           |
|                |                | (S) | 9.20                            | 8.00                                                              | 0.38                                                                     | 112.50                                                     | 75.00                                                                           |
|                | –2.0 MPa       | (R) | 3.50                            | 1.05                                                              | 0.30                                                                     | 5.40                                                       | 22.50                                                                           |
|                |                | (S) | 6.20                            | 2.00                                                              | 0.16                                                                     | 15.00                                                      | 12.40                                                                           |



The photosynthetic apparatus is sensitive to dehydration. Drought stress has a direct effect on carboxylating enzymes. The activities of enzymes RuBP carboxylase and PEP carboxylase decreased in the leaves of plants subjected to drought (Kaiser, 1987; Du et al., 1996). In sugarcane leaves, a decrease in the leaf water potential up to  $-0.37$  and  $-0.85$  MPa led to about a two to nine times decrease in the activities of RuBP carboxylase, PEP carboxylase, fructose-1,6-bisphosphatase, NADP malic enzyme, and orthophosphate dikinase, leading to an overall decreased rate of photosynthesis (Du et al., 1996). Drought stress alters carbon partitioning in plant parts owing to an alteration in the activities of sugar-metabolizing enzymes. In the leaves of sorghum plants, drought condition reduces sucrose formation owing to an inhibition in the activities of fructose-1,6-bisphosphatase and sucrose phosphate synthase (SPS) (Reddy, 1996). However, in potato tubers, a moderate drought stress leads to an activation of SPS and a stimulation of sucrose synthesis (Geigenberger et al., 1997). A more extreme drought stress in potato tubers leads to a further alteration in carbon partitioning, because it inhibits the activities of one or more of the enzymes involved in the terminal reactions of starch synthesis (Geigenberger et al., 1997).

Drought stress leads to oxidative damage in plants by inducing the production of active oxygen species and decreasing the activities of the antioxidant enzymes catalase, peroxidase, and SOD (Mali et al., 1980; Gogorcena et al., 1995). An increase in the capacity of the ascorbate regeneration system in cells by a *de novo* synthesis of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and GR is one of the primary responses of plants to water deficit so as to mitigate oxidative stress (Boo and Jung, 1999; Sharma and Dubey, 2005a). Ascorbate peroxidase (APX) serves as an important component of the antioxidative defense system under drought stress in plants (Sharma and Dubey, 2005a). An examination of the involvement of activated oxygen in the drought-induced damage of pea nodules indicated that drought stress ( $-2.03$  MPa) caused a decrease in the activities of catalase (25%), APX (18%), DHAR (15%), GR (31%), and SOD (30%) with a simultaneous decrease in the contents of ascorbate (59%), reduced glutathione (57%), and oxidized glutathione (38%) (Gogorcena et al., 1995). The overproduction of antioxidant enzymes in order to scavenge reactive oxygen species (ROS), which are greatly produced under drought, provides an elegant approach to engineer plant species for drought stress tolerance. Transformed rice plants expressing pea manganese SOD have been shown to be more resistant to drought stress (Wang et al., 2005) compared with normal plants.

Levels of many enzymes increase under drought stress. Many hydrolytic enzymes show an increased activity in drought-stressed tissues. The  $\alpha$ -amylase activity increased under drought stress, which was responsible for increased starch hydrolysis *in vivo*, leading to increased levels of sugars and a decreased level of starch in drought-stressed tissues (Hsiao, 1973). Proteases have been shown to be induced under drought stress. A thiol protease in pea and two cysteine proteinases in *Arabidopsis* have been identified, which are induced under water deficit (Bray, 1995). Certain hydrolytic as well as oxidative enzymes show different behaviors in the crop cultivars differing in drought stress tolerance. While investigating the behavior of drought-resistant and drought-sensitive *Z. mays* cultivars for protease activity under drought stress, Thakur and Thakur (1987) observed an increasing trend in the protease activity with an increasing osmotic potential in the resistant cultivar Ageti-76, whereas in the sensitive cultivar Vijay, they observed a decreased protease activity under severe drought stress. While studying the behaviors of certain hydrolytic and oxidative enzymes in the leaves of drought-stressed rice plants of the two genotypes differing in stress tolerance, Goyal and Kochhar (1988a) observed that protease, ribonuclease, peroxidase, and IAA (indole acetic acid) oxidase activities were inhibited by drought stress. However, the activity of ascorbic acid oxidase increased in both sets of cultivars. Different responses for these enzymes were observed for the two sets of cultivars differing in stress tolerance.

In certain plant species, the increased synthesis of sucrose (Pelah et al., 1997) and proline (Dubey and Pessarakli, 1995) occurs under drought stress owing to a stress-induced increase in the activities of the enzymes synthesizing these metabolites. In *P. popularis* plants, the accumulation of sucrose accompanied by the increasing activity of its synthetic enzyme SS occurs under

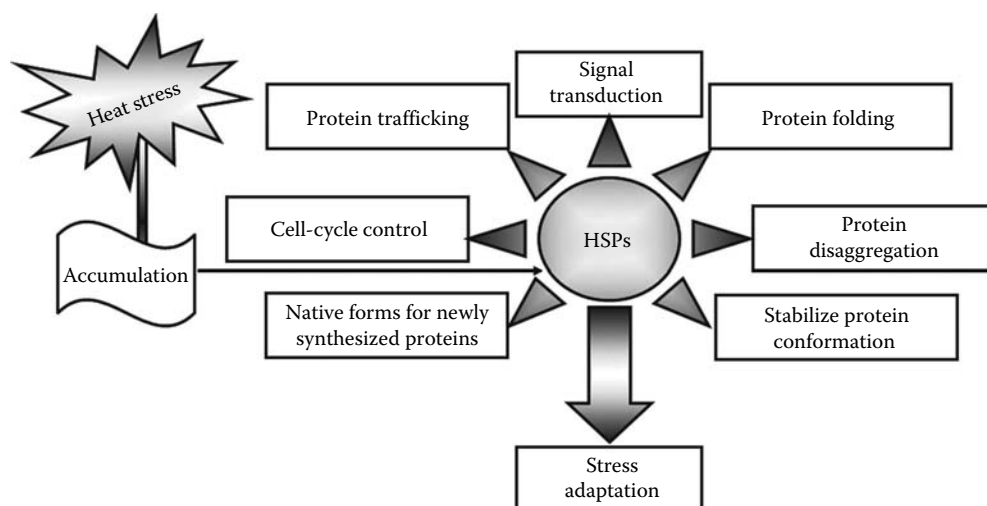
water deficit (Pelah et al., 1997). The activity of the enzyme  $\Delta^1$ -pyrroline-5-carboxylase synthetase (P5CS), which is involved in the biosynthesis of proline, increases in rice seedlings under dehydration (Igarashi et al., 1997). In cotton plants, the tolerant genotype showed higher P5CS activities under drought stress than those of the drought-sensitive genotype (Parida et al., 2008). *Petunia* plants transformed by the *P5CS* gene from *A. thaliana* L. (*AtP5CS*) or from *O. sativa* L. (*OsP5CS*) tolerated 14 days of drought stress (Yamada et al., 2005).

Different behaviors of certain oxidative enzymes have been observed depending on the different methods of creating drought as well as on the plant parts studied. The activities of IAA oxidase and peroxidase increased in etiolated drought-stressed seedlings of winged bean (*Psophocarpus tetragonolobus* L.) and amaranthus (*Amaranthus caudatus*) plants, whereas the decreased activities of the two enzymes were observed in drought-stressed green seedlings (Goyal and Kochhar, 1988b). The higher activities of oxidative enzymes under drought stress are possibly due to a gradual shift of reductive metabolism to oxidative metabolism under these conditions. While studying oxidative processes in rice plants differing in drought stress tolerance, Lodh et al. (1977) observed an increased activity of peroxidase in drought-tolerant cultivars Lalnakanda-41 and T(N) 1×T.65 as well as the drought-sensitive cultivar CO-13. These investigators observed an increased catalase activity in the drought-sensitive rice cultivar CO-13 but not in the drought-tolerant cultivars. The polyphenol oxidase activity has been reported to increase in the leaves and roots of the drought-sensitive rice cultivar CO-13, whereas, in drought-tolerant cultivars Lalnakanda-41 and T(N) 1×T.65, the activity of the enzyme decreased in the leaves as well as in the roots under drought stress (Lodh et al., 1977). These observations suggest that drought stress leads to changes in the levels of various enzymes in stressed plant parts and that the effects of stress depend on the properties of the enzymes, the severity of the stress, and the organs of the plants studied.

### 19.2.3 HEAT STRESS

High-temperature or heat stress adversely affects plant growth and the yield in many areas of the world. Some plants can survive when the temperature exceeds even 20°C above ambient, whereas in most of the field crops, temperatures above 40°C cause heat injury, severely limit photosynthesis, and alter protein metabolism by causing protein breakdown, protein denaturation, enzyme inactivation, and other effects. The heat stress response is characterized by inhibition of normal transcription and translation, higher expression of HSPs, and induction of thermotolerance (He et al., 2005). Protein degradation involving various proteases is also important in regulating plant responses to heat stress. The identification of stress-responsive proteins and pathways has been facilitated by increasing the number of tools and resources, including two-dimensional electrophoresis and mass spectrometry, and the rapidly expanding nucleotide and amino acid sequence databases (Huang and Xu, 2008).

When plants are subjected to heat treatments, beyond optimum growth temperatures, normal protein synthesis declines owing to a coordinate loss of the translational efficiency of most mRNAs and an enhanced synthesis of stress-related proteins (Iba, 2002). Immediately after exposure to high temperatures and perception of signals, changes occur at the molecular level, altering the expression of genes and accumulation of transcripts, thereby leading to the synthesis of stress-related proteins as a stress tolerance strategy (Wahid et al., 2007). Most of the available heat-related proteomic studies have focused on a more descriptive overview of proteins that occur in aboveground parts of the plant. Root thermotolerance at the proteomic level has not been well investigated, and the molecular basis is poorly known (Huang and Xu, 2008). Proteome profiling of *Populus euphratica* Oliv. upon heat stress revealed short-term upregulated proteins related to membrane destabilization and cytoskeleton restructuring, sulfur assimilation, thiamine and hydrophobic amino acid biosynthesis, and protein stability. Long-term upregulated proteins were involved in redox homeostasis and photosynthesis. Late downregulated proteins were involved mainly in carbon metabolism (Ferreira et al., 2006).



**FIGURE 19.5** Roles of HSPs in plants under heat stress. HSPs take part in signal transduction, protein folding, protein disaggregation, stabilization of protein conformation, control of cell cycle, and protein trafficking, and assist newly synthesized and newly translocated proteins to achieve their native forms, and thus confer an enhanced survival value to the plants against heat stress.

It is believed that heat tolerance in plants is associated with the synthesis of HSPs, which protect plants from otherwise nonpermissive temperatures and provide them with an endogenous protection system for thermotolerance. The synthesis of HSPs occurs in diverse plant species when they are exposed to temperatures 10°C–15°C above ambient temperatures (Cooper and Ho, 1983; Heikkila et al., 1984; Kanabus et al., 1984; Kee and Nobel, 1986; Mansfield and Key, 1988; Singla and Grover, 1994; Cordewener et al., 1995; Waters et al., 1996). Crop plants, such as maize, soybean, cowpea, and wheat, start synthesizing HSPs in the tissues with a rise in tissue temperatures beyond 32°C–33°C (Vierling, 1991; Efeoglu and Terzioglu, 2007). The induction of HSP synthesis parallels the increase in temperature. It has been observed that the exposure of plants to higher temperatures of heat shock leads to the stability as well as the rapid induction of specific mRNAs related to specific HSPs (Sullivan et al., 1990). The HSPs range in molecular mass from about 10 to 200 kDa (Schoffl et al., 1999). The phenomenon of heat-shock response (HSR) is conserved among all biological organisms. Although HSPs provide the molecular basis for thermotolerance, whether they act directly in signal transduction or induce the synthesis of secondary agents involved in protection is not yet clear. The HSPs are proposed to act as molecular chaperones (Gething, 1997). They function in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions (Wang et al., 2004). The possible different roles of HSPs in plants under heat stress have been shown in Figure 19.5. The HSPs take part in protein disaggregation, stabilization of protein conformation, control of cell cycle, and protein trafficking, and assist newly synthesized and newly translocated proteins to achieve their native forms. The tolerance conferred by HSPs results in improved physiological phenomena like photosynthesis, assimilate partitioning, water and nutrient use efficiency, membrane stability, etc. (Camejo et al., 2005; Ahn and Zimmerman, 2006; Momcilovic and Ristic, 2007).

### 19.2.3.1 Synthesis of Heat-Shock Proteins

The synthesis of HSPs occurs in plant cell cultures undergoing thermoadaptation or intact plants subjected to heat stress (Vierling, 1991). It has been shown that not only heat stress but other conditions, such as treatment with arsenite, heavy metals, ethanol (Vierling, 1991), ABA, drought stress, and wounding (Heikkila et al., 1984), also induce the expression of some of the HSP mRNAs and

the synthesis of HSPs in plants. This shows that the synthesis of HSPs can be induced even in the absence of heat stress, and high-temperature protection can be provided without prior heat shock.

He and coworkers (2005) investigated the effects of heat acclimation and sudden heat stress on protein synthesis in creeping bentgrass (*Agrostis palustris* Huds.) and observed that heat acclimation induced the expression of some of the HSPs with mol. wt. 57 and 54 kDa, in the cytoplasmic fraction, which were not present in unacclimated plants under heat stress. However, HSPs of 23, 36, and 66 kDa were induced by both sudden and gradual exposure to heat stress. Newly synthesized HSPs during heat acclimation appear to be associated with the enhanced thermotolerance of creeping bentgrass (He et al., 2005). A two-dimensional protein analysis revealed stronger and more diverse HSR in wheat seedlings exposed to 37°C for 8 h compared to other treatments like 37°C for 4 h, 45°C for 8 h, 45°C for 4 h, and 45°C for 2 h. Five protein spots, ranging from 6 to 7.8 pI and from 27 to 31.7 kDa molecular weight, were expressed when seedlings were exposed to 37°C for 2 h and continued at 37°C and 45°C for all exposure times. This suggests that these early proteins and other newly synthesized proteins may have protective effects at 37°C and 45°C and appear to help in achieving a healthy growth to plants during the recovery period (Efeoglu and Terzioglu, 2007). Cytoplasmic distribution and subcellular localization of HSPs indicate that they remain either specifically associated with various organelles, such as nuclei, chloroplasts, mitochondria, PM, or as cytoplasmic aggregates distinct from ribosome granules (Mansfield and Key, 1988; Vierling, 1991). When the tissue temperature exceeds 32°C–33°C, HSPs are typically seen. The appearance of these proteins has been positively correlated with an enhanced thermotolerance, and, as well, it also provides a certain level of cross protection to other kinds of stresses (Artlip and Funkhouser, 1995).

In carrot cells, the heat-shock treatment causes an inhibition of protein synthesis with the simultaneous appearance of new proteins (Pitto et al., 1983). In carrot cells, patterns of these newly synthesized proteins become different depending on the growth stages of cells and culture conditions. It was shown by Kanabus et al. (1984) that tobacco cell suspensions synthesize HSPs in different phases of the growth cycle. When maize tissues are exposed to heat shock, they show the induction of HSP mRNA and the synthesis of a set of 10 HSPs (Cooper and Ho, 1983; Heikkila et al., 1984). Different tissues of the same plant, as well as different developmental stages of the tissues, show different patterns of HSPs (Burke et al., 1985).

In field-grown cotton (*Gossypium hirsutum* L.) plants, when the temperature reaches 40°C for a few weeks, the synthesis of eight unique polypeptides of 100, 94, 89, 60, 58, 37, and 21 kDa occurs (Burke et al., 1985). These polypeptides accumulate in dryland plants that have a canopy temperature of 40°C and are absent in irrigated plants that have a 30°C canopy temperature. A group of 11 newly synthesized polypeptides accumulated in laboratory-grown heat-shocked cotton plants, as revealed by autoradiography of radiolabeled polypeptides. Of these 11 polypeptides, 8 appeared to be similar to those of heat-shocked field-grown cotton plants (Burke et al., 1985). These results suggest that dryland crops synthesize HSPs in substantial levels in response to high temperatures.

Many desert succulent plants have been shown to accumulate HSPs when day and night air temperatures are raised from 30°C and 20°C to 50°C and 40°C, respectively (Kee and Nobel, 1986). The pattern of accumulation of HSPs is species specific in these plants; however, a unique 25–27 kDa protein accumulated in all species examined, which appeared to be associated with the thermotolerance in these plants (Kee and Nobel, 1986). An elevation of the culture temperature to 32°C for 8 h leads to an irreversible *de novo* synthesis of a number of HSPs of a 70 kDa class, HSP68 and HSP70 in *B. napus* (Cordewener et al., 1995). Five-day-old rice seedlings, when subjected to a temperature stress of 45°C for 1–2 h, synthesized and accumulated a 104 kDa polypeptide, which constituted about 0.4% of the total soluble protein fraction (Singla and Grover, 1994).

Among the wide range of HSPs that accumulate under heat shock, some are specifically associated with organelles, including the nucleus, nucleolus, chloroplast, mitochondria, and PM. Certain other HSPs are found to be associated with the ribosome or they remain as aggregates in the cytoplasm (Mansfield and Key, 1988). In pigeon pea (*C. cajan*) plants, HSPs of 18, 20, 22, and 24 kDa are found to be associated with mitochondrial and membrane fractions, whereas the 60, 70, and

81 kDa proteins are found in the soluble fraction (Kishore and Upadhyaya, 1994). In the mitochondria of pea plants, a novel 22 kDa protein accumulates in the matrix when the normal growth temperature is shifted from 25°C to 40°C (Lenne and Douce, 1994).

The effect of heat stress on soluble proteins extracted from leaf tissues of wheat cultivars differing in sensitivity to high temperature was examined by two-dimensional gel electrophoresis. Twenty-two of 31 differentially expressed proteins were detected as newly synthesized LMW or small HSPs (LMW HSPs, sHSPs). The number of the sHSPs in heat-tolerant cultivars was higher than in the heat-sensitive cultivars. Some of the sHSPs were cultivar specific (Yildiz and Terzi, 2008). These proteins were suggested to play an important role in acquiring thermotolerance. Rice seedlings subjected to high temperatures showed 73 differentially expressed proteins, among which 48 proteins were identified, and it was observed that a group of sHSPs were newly induced in rice by heat stress (Lee et al., 2007). Among these sHSPs, an LMW mitochondrial sHSP was validated further by the western blot analysis (Lee et al., 2007).

### 19.2.3.2 Types of Heat-Shock Proteins

Heat shock induces the synthesis of a wide range of HSPs in plants. A general system of classification for these proteins is based on their molecular weights and their localization in the cell. There are five major conserved families of HSPs/chaperones: the HSP100/Clp family, the HSP90 family, the HSP70/DnaK family, the chaperonins (GroEL and HSP60), and the small HSP (sHSP) family (Huang and Xu, 2008). HSP100/Clp family chaperones are reported to be induced by different environmental stresses (Wang et al., 2004). A reduced thermotolerance was observed in transgenic lines, expressing an antisense construct of tomato chloroplast-localized HSP100/ClpB (Yang et al., 2006).

Many HSPs, like HSP60, HSP70, and HSP90, are present as constitutive proteins in the cytoplasm as well as other in organelles, like mitochondria and chloroplasts of plants in nonstressed conditions, and their level increases under heat shock. Studies indicate that HSPs function in a fashion similar to molecular “chaperons” and assist the self-assembly of nascent polypeptides into their correctly folded tertiary structures, and also prevent the formation of an aggregation of nonfunctional proteins resulting from heat denaturation (Artlip and Funkhouser, 1995). Especially, small HSPs that range in sizes from 12 to 40 kDa and are encoded by six nuclear gene families, accumulate to high levels in response to heat stress and bind partially denatured proteins, preventing irreversible protein inactivation and aggregation, and thus contribute to the development of thermotolerance (Waters et al., 1996). According to Harrington et al. (1994), HSPs have a possible function in signal transduction involving protein kinases and heat shock-induced calmodulin-binding proteins.

Transgenic tobacco plants constitutively expressing elevated levels of NtHSP70–1 showed inhibition in fragmentation and degradation of nuclear DNA during heat stress. In addition, seedlings constitutively overexpressing NtHSP70–1 grew as healthy plants, whereas antisense seedlings resulted in death after heat/drought stress (Cho and Choi, 2009).

### 19.2.4 CHILLING

Chilling is one of the most severe constraints limiting crop productivity. Low environmental temperatures lead to chilling injury in plants and result in the loss of PM integrity, irreversible and proportional loss of proteins from the cell, and ultimately the death of the cell (Levitt, 1980; Stuiver et al., 1988). According to Levitt (1980), freezing-induced dehydration within the cell leads to the aggregation of proteins owing to the formation of disulfide bonds as well as the denaturation of soluble proteins. The synthesis of many key enzyme proteins decreases when plants are exposed to low temperatures (Bruggemann et al., 1994; Matsuba et al., 1997), and the synthesis of certain specific proteins is induced (Griffith et al., 1997).

Karimzadeh and coworkers (2006) observed changes in the electrophoretic pattern of the water-soluble proteins from spring and winter wheat cultivars, and pointed out the accumulation of stress proteins in leaves on exposure to freezing temperature. When the biosyntheses of stress proteins

were analyzed after a short-term low-temperature treatment in the leaf cells of *Brassica campestris* var. *olifera* and *Amaranthu caudatus* L. (ruderals) and *Rumex patientia* L.  $\times$  *Rumex tianshanicus* A. Los. (stress tolerant), differences in protein patterns were observed in control and treated samples (Kosakivska et al., 2008). A short-term cold stress caused a substantial increase in the protein content in stress-tolerant plants. This was suggested to be due to the activation of a stress response mechanism, which in turn upregulates protein synthesis (Kosakivska et al., 2008). Many enzymic proteins, especially those of the carbon assimilation pathway, are extremely sensitive to chilling. The key photosynthetic enzyme of  $C_3$  plants, RUBISCO, which constitutes about 60% of soluble proteins, undergoes changes in structure, conformation, and properties at low temperatures (Stuiver et al., 1988; Bruggemann et al., 1994). In *Zoysia japonica* plants, a drastic decline in the level of the  $C_4$ -cycle enzymes phosphoenol pyruvate carboxylase (PEPC) and phosphoenolpyruvate carboxykinase (PCK) takes place during exposure to low temperature. In *Lycopersicon esculentum* and *Z. mays* plants, chilling stress results in an irreversible loss of RUBISCO and stromal fructose-1,6-bisphosphatase activities (Bruggemann et al., 1994). In the conifer *Pinus sylvestris*, the contents of the D1 protein of the photosystemII (PSII) reaction center and of the PSII light-harvesting complex (LHCII) proteins decline under low-temperature stress (Ottander et al., 1995). Similarly, the SH-rich enzyme GR becomes partially inactivated by freezing (Guy and Carter, 1984). It is suggested that the activity loss of many enzymes on chilling is as a result of a modification of sulfhydryl groups or other side chains of the protein (Bruggemann et al., 1994).

The chilling of etiolated hypocotyls of *Vigna radiata* L. for 1 or 2 days induced the synthesis of two soluble proteins (82 and 74 kDa) and one membrane protein (80 kDa). Moreover, three HSPs that were induced by heat stress (41°C, 4 h) had the same electrophoretic mobilities as those of the proteins induced directly or indirectly by the chilling treatment (Kawata and Yoshida, 1988). Protein expression patterns of rice roots in response to chilling stress revealed 27 upregulated proteins. Together with the previously identified cold stress-responsive proteins, a group of novel proteins were identified including acetyl transferase, phosphogluconate dehydrogenase, NADP-specific isocitrate dehydrogenase, fructokinase, PrMC3, putative alpha-soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein, and glyoxalase 1. These proteins are involved in several cellular processes, including energy production and metabolism, vesicular trafficking, and detoxification (Lee et al., 2009). Using two-dimensional electrophoresis, Hashimoto and Komatsu (2007) detected the synthesis of four new proteins in rice in response to cold stress. A comparative proteomic analysis has provided new insights into chilling stress responses in rice. Yan et al. (2006) using a mass spectrometry analysis identified 85 differentially expressed proteins, including well-known cold-responsive proteins, such as enolase, RcbL, RcbA, APX, and HSPs, and several novel cold-responsive proteins, such as 2-Cys peroxiredoxin, armadillo repeat-containing protein, and putative nascent polypeptide-associated complex  $\alpha$  chain. sHSPs have been suggested to play relevant roles in the acquisition of freezing tolerance in *Castanea sativa* (Lopez-Matas et al., 2004).

#### 19.2.4.1 Cold Acclimation

When plants are exposed to low nonfreezing temperatures for a few hours or a day, certain new sets of proteins are synthesized, and these plants develop the capacity to adapt to subsequent chilling or freezing temperatures. Such a mechanism of adaptation is known as cold acclimation. Generally, temperatures from 4°C to 15°C are considered to be chilling, whereas a temperature below 4°C is considered to be freezing (Artlip and Funkhouser, 1995). CA results in altered gene expression, leading to a synthesis of specific proteins and certain enzymes, which are responsible for the development of freezing tolerance (Dhindsa and Mohapatra, 1990; Antikainen and Griffith, 1997; Griffith et al., 1997). Several preexisting proteins abundant in the tissues of plants grown under normal temperatures, decline on exposure to low temperatures. However, many new transcripts and polypeptides are synthesized (Meza-Basso et al., 1986; Guy and Haskell, 1987; Griffith et al., 1997; Yan et al., 2006; Hashimoto and Komatsu, 2007), which appear to play a major role in the acclimation of plants to freezing temperatures (Griffith et al., 1997).

Plants differ in their capacity to tolerate low temperatures. In many crop plants, freezing tolerance can be induced by exposure to low nonfreezing temperatures. Freezing-tolerant or cold-acclimated plants possess new proteins, which are not present in normal or non-acclimatized plants. Uemura and Yoshida (1984), while studying CA in winter rye (*Secale cereale* L.) seedlings, observed that more than 20 proteins disappeared in the PM during acclimation, and the concentration of 11 proteins increased, whereas 26 new proteins were synthesized. In young rapeseed *B. napus* seedlings, a 48 h exposure to nonchilling temperatures induces important changes in gene expression. The synthesis of specific polypeptides is increased with a concomitant increase in respective mRNA levels, whereas the synthesis of six polypeptides is suppressed with the degradation of their corresponding mRNAs (Meza-Basso et al., 1986). Transcripts of a gene encoding a putative cell wall PM linker proline-rich protein has been isolated by Goodwin et al. (1996) from *B. napus* leaves, which are specifically expressed in leaves on exposure to low temperature. This indicates that an increase in the level of specific mRNA transcripts and their corresponding proteins is correlated with improved freezing tolerance (Meza-Basso et al., 1986). The CA of rapeseed seedlings leads to a decreased level of mRNA for the small RUBISCO subunit as well as its reduced synthesis (Meza-Basso et al., 1986). In cold-sensitive rice (*O. sativa* L.) plants, CA leads to suppression as well as induction in gene expression, resulting in a decreased level of certain proteins and an increased synthesis of other specific proteins as well as corresponding mRNAs (Hahn and Walbot, 1989). Hahn and Walbot (1989), while studying the effect of cold treatment on the pattern of protein synthesis in rice leaves, detected several novel proteins of 95, 75, 25, and 21 kDa that were synthesized during 1–7 days of 11°C and 6°C cold treatment. Since these proteins were cold specific, other stresses, such as drought stress, salinity, and acid treatment, could not induce the synthesis of such proteins.

Osmotin has also been proposed to play a role in CA (D'Angeli and Altamura, 2007). The over-expression of a tobacco osmotin gene in olive tree demonstrated its involvement in CA-related programmed cell death, blocking cold-induced calcium signaling, and cold-induced cytoskeleton alterations. However, the underlying mechanism of the protection provided by osmotin to tolerate cold stress is still unclear. In freezing-tolerant cereal plants, such as rice, wheat, and barley, antifreeze proteins were synthesized during CA, which appear to play a significant role in increasing freezing tolerance (Antikainen and Griffith, 1997; Griffith et al., 1997). Six antifreeze proteins that have the unique ability to absorb onto the surface of ice and inhibit its growth have been isolated from the apoplast of winter rye leaves where ice forms at subzero temperatures (Griffith et al., 1997). Among the rye antifreeze proteins, two are endoglucanase-like, two chitinase-like, and two thaumatin-like proteins (TLPs) (Griffith et al., 1997). The accumulation of antifreeze proteins is not a general response to all plants, but it is a specific response that is important in the antifreeze tolerance for certain plants. Transgenic tobacco plants expressing carrot antifreeze protein showed an increased freeze resistance and an inhibition of electrolyte leakage from cold-stressed cells (Worrall et al., 1998; Fan et al., 2002). Antarctic hair grass (*Deschampsia antarctica* E. Desv.), the only grass indigenous to Antarctica, has well-developed freezing tolerance, strongly induced by CA. In response to low temperatures, *D. antarctica* expresses a potent recrystallization inhibition (RI) activity that inhibits the growth of small ice crystals into potentially damaging large ones. It is proteinaceous in nature and remains localized in the apoplasm. A gene family from *D. antarctica* encoding putative homologs of an ice recrystallization inhibition protein (IRIP) has been isolated and characterized. *D. antarctica* IRIP (DaIRIP) transcript levels are greatly enhanced in leaf tissues following CA. Transgenic *A. thaliana* expressing a DaIRIP has a novel RI activity, and the purified DaIRIP when added back to the extracts of leaves from non-acclimated *D. antarctica*, can reconstitute the activity found in acclimated plants (John et al., 2009).

In certain plants, such as citrus (Durham et al., 1991) and some herbaceous species (Guy et al., 1988), a very high-molecular-weight protein has been identified that is specifically synthesized under CA. Durham et al. (1991), while comparing polypeptide patterns resulting from *in vitro* translations of total RNA isolated from cold-acclimatized and non-cold-acclimatized leaf tissues of cold-sensitive *Citrus grandis* plants, observed a 160 kDa polypeptide in cold-acclimatized leaves that

was not present in non-cold-acclimatized citrus leaves. This 160kDa unique polypeptide has also been detected in cold-acclimatized spinach and sweet orange, *C. sinensis* (Guy et al., 1988).

In *A. thaliana*, two glycine-rich proteins, MSACIA and MSACIB, accumulate during CA. The timing and localization of the expression of these two proteins are different, and the differential expression involves both transcriptional and posttranscriptional events (Ferullo et al., 1997). Comparisons among different cultivars of *A. thaliana* suggest that low freezing tolerance is associated with the failure to accumulate these proteins. Mantyla et al. (1995) have shown the accumulation of ABA-responsive RAB18 and LTI18 proteins in *A. thaliana* plants under CA. In floral buds of a woody perennial blueberry (*Vaccinium*), three different dehydrin-like lysine-rich proteins of 65, 60, and 14kDa accumulated in response to chilling (Muthalif and Rowland, 1994). Dehydrins are typically induced in response to abiotic stresses that impose cellular dehydration. As extracellular freezing results in cellular dehydration, the accumulation of dehydrins and the development of desiccation tolerance are believed to be key components of the CA process. The accumulation of high levels of dehydrin transcripts has been observed in field-grown freezing-tolerant brome-grass (*Bromus inermis* L.) and rye (*S. cereale*) plants (Robertson et al., 1994). Field-acclimated plants accumulating high levels of dehydrin transcripts and proteins have been regarded as being more freeze tolerant (Robertson et al., 1994; Arora et al., 1997). Several studies have demonstrated an increased accumulation of dehydrin transcripts or proteins in plants during the coldest months (Welling et al., 2004; Wisniewski et al., 2006). Peng and coworkers (2008) observed that RcDhn5, one of the dehydrins from *Rhododendron catawbiense* leaf tissues, encodes an acidic, SK2-type dehydrin, and is upregulated during seasonal CA and downregulated during spring deacclimation (DA). Data from *in vitro* partial water loss assays indicate that purified RcDhn5 protects enzyme activity against a dehydration treatment and that this protection is comparable with acidic SKn dehydrins from other species. *Arabidopsis* plants overexpressing RcDhn5 exhibited an improved “constitutive” freezing tolerance compared with the control plants (Peng et al., 2008). To understand the development of CA in highbush blueberry plants (*Vaccinium corymbosum* L.), Kikuchi and Masuda (2009) investigated seasonal changes in the protein compositions of current-year and overwintered shoots. Amounts of a few proteins increased in autumn, in association with the enhancement of cold hardiness. Of these proteins, 65 and 60kDa proteins were confirmed to be dehydrins by western blotting. While the levels of most of the accumulated proteins decreased in April, a 27kDa protein maintained its level in the overwintered stem during spring. The amino acid sequence deduced from a cDNA for this protein showed significant similarities with known chitinases. The accumulation of chitinase was concluded to be involved in tolerance to low temperature in winter or unseasonably low temperature in spring (Kikuchi and Masuda, 2009). The expression of a *Dhn24* gene encoding an SK<sub>3</sub>-type DHN24 dehydrin from cold-acclimated species *Solanum sogarandinum* showed a significantly reduced chilling injury value in transgenic cucumber lines (Yin et al., 2006). To elucidate the contribution of dehydrins to freezing stress tolerance in *Arabidopsis*, transgenic plants overexpressing multiple dehydrin genes were generated. Chimeric double constructs for expression of RAB18 and COR47 (pTP9) or LTI29 and LTI30 (pTP10) were made by fusing the coding sequences of the respective dehydrin genes to the cauliflower mosaic virus 35S promoter. The overexpression of the chimeric genes in *Arabidopsis* resulted in the accumulation of the corresponding dehydrins to levels similar or higher than in cold-acclimated wild-type plants. Transgenic plants exhibited lower LT50 values and showed improved survival when exposed to freezing temperatures compared to the control plants. In transgenic plants, post-embedding immunoelectron microscopy of high-pressure frozen, freeze-substituted samples revealed a partial intracellular translocation of the acidic dehydrin LTI29 from cytosol to the vicinity of the membranes during CA. This study provides evidence that dehydrins contribute to freezing stress tolerance in plants and suggests that this could be partly due to their protective effect on membranes (Puhakainen et al., 2004).

There does not appear to be any uniform pattern of protein synthesis among plant species during CA. This implies that CA-induced proteins are not highly conserved as HSPs. A characteristic feature of CA-induced proteins is that some of the synthesized proteins are transient, whereas others



are stable, the synthesis of which continues for weeks (Guy and Haskell, 1987). CA and overwintering of herbaceous plants are energetically expensive and are dependent on functional plastid metabolism. Cold shock (1 day) has been shown to have an effect on *Arabidopsis* plastid proteomes, while short-term (10 days) acclimation results in major changes in the stromal but few changes in the lumen proteome (Goulas et al., 2006). Long-term acclimation (40 days) results in the modulation of the proteomes of both compartments, with new proteins appearing in the lumen, and further modulations in protein abundance occur in the stroma. Forty-three differentially displayed proteins were observed that appeared to participate in photosynthesis, other plastid metabolic functions, hormone biosynthesis, and stress-sensing and signal transduction (Goulas et al., 2006).

Some plants are able to adapt to cold through mechanisms based on protein synthesis due to gene induction. The best understood genetic pathway leading to gene induction upon a temperature downshift is based on C-repeat-binding factors (CBF) activating promoters through the C-repeat (CRT) cis-element. Such activation of transcription factors suggests that cold, as a signal, has been transduced into the cells (Ruelland et al., 2009). The overexpression of the *Arabidopsis* transcriptional activator *CBF1* (C-repeat/drought-responsive element [CRT/DRE] binding factor 1) induces *cor* (cold-regulated) genes and enhances freezing tolerance in non-acclimated *Arabidopsis* plants (Jaglo-Ottosen et al., 1998). The expression of barley *HvCBF4* was shown to enhance tolerance to abiotic stress in transgenic rice (Oh et al., 2007). A cis-acting promoter element, DRE, plays an important role in regulating gene expression in response to abiotic stresses. The overexpression of the cDNA encoding DREB1A in transgenic plants activated the expression of many of the stress-tolerance genes under normal growing conditions and resulted in improved tolerance of plants toward drought, salt loading, and freezing (Liu et al., 1998; Kasuga et al., 1999, 2004; James et al., 2008). Similarly, Gutha and Reddy (2008) observed an increased tolerance of transgenic tobacco plants overexpressing *OsDREB1B* under various abiotic stresses, including osmotic stress and freezing stress.

The inheritance of freezing tolerance appears to be a multigenic phenomenon, and the precise function of the proteins encoded by these genes is not fully known. Both transcriptional and post-transcriptional controls have been shown to be involved in the expression of these genes (Hughes and Dunn, 1996).

#### 19.2.4.2 Absciscic Acid and CA

It has been observed that exogenous ABA induces freezing tolerance in many plant species (Dhindsa and Mohapatra, 1990; Tseng and Li, 1991), although the physiological basis of this phenomenon is poorly understood. In certain plants, an increase in the exogenous ABA level is observed following CA (Tseng and Li, 1991; Artlip and Funkhouser, 1995; Pagter et al., 2008). Plantlets of potato (*Solanum commersonii*) stem culture, when treated with ABA for 14 days, develop cold tolerance with the concomitant induction of 30 polypeptides (Tseng and Li, 1991). It is highly unlikely that ABA regulates all the genes associated with CA; however, it definitely regulates many of the genes associated with an increase in freezing tolerance (Gusta et al., 2005). Evidence suggests that there may be ABA-dependent and ABA-independent pathways involved in the acclimation process. Several specific translatable mRNA populations and their *in vivo* translation products have been identified following the ABA treatment of potato plantlets (Robertson et al., 1994). It is suggested that ABA alters gene expression, leading to the development of cold hardiness (Mohapatra et al., 1988) by the synthesis of certain specific polypeptides that are similar to some of the polypeptides synthesized during CA (Tseng and Li, 1991; Mantyla et al., 1995). The ABA has been shown to induce the synthesis of certain polypeptides that are not synthesized in CA tissues (Tseng and Li, 1991). A comparative study of CA-induced proteins and ABA-induced proteins suggests that both CA and ABA induce the synthesis of specific and certain common proteins. This also suggests that the full development of cold tolerance requires the synthesis of complete sets of CA-induced proteins, because certain genes, in addition to those responsive to ABA, are involved in the development of maximum freezing tolerance (Dhindsa and Mohapatra, 1990).

### 19.2.5 ANAEROBIC STRESS

Anaerobic stress is generally caused by excessively wet soil or flooding conditions. Anaerobiosis affects plant metabolism as a result of a low oxygen concentration in the rooting medium. Plants adapting to anaerobic stress switch from oxidative to fermentative carbohydrate metabolism (Ricard et al., 1991). Under anaerobic stress, normal protein synthesis is suppressed, associated with the loss of polysomes, and gene expression alters, leading to synthesis of specific sets of novel polypeptides and proteins commonly known as transition polypeptides (TPs) and anaerobic polypeptides (ANPs). The repression of preexisting aerobic proteins and the synthesis of new proteins appear to be the immediate biochemical response of anaerobiosis (Mohapatra et al., 1988; Subbaiah and Sachs, 2003; Olgun et al., 2008). Most of the studies related to protein synthesis under anaerobic conditions have been performed in maize (Sachs et al., 1980), rice (Ricard et al., 1991), and *Arabidopsis* (Dolferus et al., 1997). Transient polypeptides are translated primarily during the first 5 h of anoxia, and they are stable and last long after their synthesis declines, whereas ANPs appear approximately after 90 min of anoxia, and their synthesis continues for several days until cell death (Artlip and Funkhouser, 1995).

A complete lack of oxygen (anoxia) leads to an immediate cessation of protein synthesis, followed by a selective synthesis of about 20 anaerobic proteins in maize seedlings. Among these are enzymes involved in glycolytic fermentative pathways needed for rescuing the cell from the resulting energy crisis and other genes that appear to function in longer-term responses, such as aerenchyma formation and root tip death. Although “aerobic” proteins continue to be synthesized under hypoxia, the majority of the “anaerobic” genes are transcriptionally and translationally induced even by a partial depletion of oxygen (hypoxia) (Subbaiah and Sachs, 2003). Because of anaerobic conditions in maize, initially the rapid synthesis of four 33 kDa TPs takes place, whereas after 90 min of anoxia, the selective synthesis of an additional 20 polypeptides occurs (Sachs et al., 1980, 1996), which represent about 70% of the total proteins synthesized during anaerobiosis (Sachs et al., 1980). Anaerobic stress-induced proteins are different from HSPs except for a few that are common to both types of stresses (Sachs et al., 1996).

Most of the ANPs are apparently involved in maintaining ATP levels in the cells. Many of these are enzymes involved in glycolysis or fermentative processes, such as ADH, lactate dehydrogenase (LDH), aldolase, enolase, glucose-phosphate isomerase, glyceraldehydes-3-phosphate dehydrogenase, pyruvate decarboxylase, and SS (Sachs et al., 1996). Among these enzymes, ADH is the best characterized. In several tissues of maize examined, *ADH* gene expression is maximal with anoxia (Artlip and Funkhouser, 1995). Similarly, in *A. thaliana*, two *ADH* genes exist: one set is strongly induced by low oxygen stress mainly in roots, whereas the other set is expressed constitutively in both roots and leaves (Dolferus et al., 1997). In maize seedlings during several days of hypoxic induction, LDH activity increases up to 3.5-fold. This increased activity is the result of increased protein levels, which can be correlated with the induction of two *ldh* transcripts of 1.3 and 1.7 kb (Christopher and Good, 1996). A proteome analysis of the coleoptile of an anoxia-tolerant rice genotype revealed that one of the proteins synthesized during anoxia was orthophosphate dikinase (PDK) that could help to generate pyrophosphate from ATP (Huang et al., 2005).

Ricard et al. (1991) observed a significant increase in the level of SS with a concomitant increase in its mRNA level in rice seedlings subjected to anaerobiosis. Unlike maize, only one SS protein exists in rice. Its synthesis is enhanced with a concomitant increase in mRNA levels under anaerobiosis (Ricard et al., 1991), which indicates that its level of control is possibly transcriptional. Two enzymes, which have different functions than ANPs, have been identified, the level of which increases in response to hypoxia. These are 1-aminocarboxylate-1-cyclopropane synthase (ACC synthase), which catalyzes the rate-limiting step in the synthesis of ethylene, and xyloglucan endotransglycosylase, which is possibly involved in aerenchyma formation during flooding (Artlip and Funkhouser, 1995; Sachs et al., 1996). When seven-day-old rice seedlings were subjected to

anaerobic stress, only minor changes in the pattern of proteins were observed in the shoots, whereas the disappearance of many protein bands was observed in the roots. Three anaerobic stress proteins (ANPs; 36, 40, and 87 kD proteins) were selectively induced in both roots and shoots of the seedlings, and a 36 kD ANP was identified as the glycolytic enzyme, GAPDH, by limited N-terminal amino acid sequencing. The activities of GAPDH in the shoots and roots increased due to stress of over 24 h and were 3.4- and 6.2-fold greater than those in nonstressed seedlings at 24 h. These results suggest that anaerobiosis induces the production of ANPs including GAPDH in the seedlings, which may allow the seedlings to survive under stress conditions (Kato-Noguchi, 2000).

In maize seedlings, it has been observed that treatment with ABA increases tolerance to anaerobic conditions (Hwang and Van Toai, 1991). Such an induction of tolerance is partly attributed to the synthesis of new proteins. It was shown by Hwang and Van Toai (1991) that cycloheximide when added together with ABA reduced the survival rate of maize seedlings. However, ABA-induced tolerance appears to be specific, because results similar to those with maize are not observed in other crops. Oxidative stress is an integral part of many stress situations including hypoxia (Blokchina et al., 2003). The appearance of additional activity bands of SOD in native gels resulting in an increase in total activity was observed in wheat roots under anoxia. Although SOD mRNA levels were diminished, the protein content of different SOD isoforms increased with the duration of anoxia. Crude subcellular fractionation experiments implied that the anoxia-responsive SOD isoforms might be plastid-associated. SOD was suggested to be a very stable enzyme, which, under anoxia, accumulates relative to the total protein content and remains active even after protein modification under severe environmental stress conditions (Biemelt et al., 2000).

### 19.2.6 PATHOGENESIS

When plants are infected with pathogens, such as bacteria, viruses, and fungi, certain novel proteins are synthesized. The host-coded proteins, which are induced by a wide range of pathogens, are commonly known as pathogenesis-related proteins. Many of the PR proteins are also induced by abnormal concentrations of plant hormones, or they are due to the presence of pollutants, such as heavy metals (Jung et al., 1995). Since these proteins are synthesized during infection, they appear to have a possible role in inducing resistance against further infection by pathogens (Tornerio et al., 1997).

The PR proteins form a heterogeneous family of plant proteins and have been grouped into different classes based on biological properties, enzyme activity, and coding sequence similarities. Antifungal proteins/PR proteins currently comprise 17 families of induced proteins including four families of chitinases, one each of 1,3-glucanases, and proteinase inhibitors, one specific peroxidase, a PR-1 family with unknown biochemical properties, the thaumatin-like PR-5 family, the birch allergen Betv1-related PR-10 family, lipid-transfer proteins, thionins, defensins, and other proteins including 2S storage albumins, and ribosome inactivating proteins (RIPs) (Selitrennikoff, 2001; Ghosh et al., 2006). Osmotin, a 26 kDa protein, initially described in tobacco suspension cultures under salinity stress, belongs to the family of class 5a PR proteins, and it has antifungal activity (Artlip and Funkhouser, 1995). The osmotin level was found to be induced in response to viral and fungal pathogen infections in tobacco and tomato (Stintzi et al., 1991; Woloshuk et al., 1991). Osmotin was also shown to inhibit the growth of a wide range of fungal pathogens including *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Vigers et al., 1992; Abad et al., 1996). The overexpression of tobacco osmotin delayed the development of late blight disease in potato (Liu et al., 1994). The expression of apoplastically secreted tobacco osmotin in cotton has been reported to confer a moderate degree of resistance against *Rhizoctonia solani* (Parkhi et al., 2009).

Chitinase and  $\beta$ -1,3-glucanases are the best characterized PR proteins. Chitinases hydrolyze  $\beta$ -1,4-acetyl glucosamine linkages of chitin polymers, which are primary constituents of fungal cell walls, whereas glucanases hydrolyze  $\beta$ -1,3-glucan residues present in fungal cell walls (Artlip and Funkhouser, 1995). Chitinases belong to four classes. Class PR-3 includes chitinases of class Ia, Ib, II, IV, VI, and VII; chitinases of class III belong to PR-8; and chitinases of class V to PR-11.

Additionally, in class PR-4, some proteins with low endochitinase activity were found among the chitin-binding proteins (Neuhaus et al., 1996). The overexpression of chitinase and/or  $\beta$ -1,3-glucanases in transgenic plants provides considerable protection against fungal pathogens. Chitinases show an increased level in many plant species, including rice (Kim et al., 2009), *Arabidopsis* (Samac and Shah, 1991), tobacco, wheat (Broekaert et al., 1988), pepper (Egea et al., 1996), tomato (Lawrence et al., 1996), and *P. vulgaris* (Dann et al., 1996), in response to infection by fungal pathogens or viruses. Chitinases have potent antifungal activity (Broekaert et al., 1988). Four isozymes of chitinase (26, 27, 30, and 32 kDa) are induced in tomato plants on infection by the fungus *Alternaria solani* (Lawrence et al., 1996). It is suggested that a higher constitutive level of chitinase and  $\beta$ -1,3-glucanase and the induction pattern of a 30 kDa chitinase isoenzyme in early blight-resistant breeding lines is related to a genetically inherited resistance of tomato to *A. solani* (Lawrence et al., 1996). A novel *Oryza grandiglumis* Chitinase IVa (*OgChitIVa*) gene was suggested to play a role in the signal transduction process in defense response against *Botrytis cinerea* in plants. It was demonstrated that the overexpression of *OgChitIVa* from *Arabidopsis* resulted in a mild resistance against the fungal pathogen, *B. cinerea*, by lowering the disease rate and the necrosis size (Pak et al., 2009).

Two class 1 PR proteins, designated as acidic PR-1 protein (PR1a1) and basic PR-1 protein (PR1b1), which are LMW proteins and are encoded by two closely related genes, have been characterized in tomato plants (Tornero et al., 1997). The expression of these two proteins is also induced by salicylic acid and ethylene. In transgenic tobacco plants infected with tobacco mosaic virus (TMV), the *PR1b1* gene is strongly activated locally in tissues undergoing a hypersensitive response but not systemically in uninoculated tissues (Tornero et al., 1997). In the primary leaves of *P. vulgaris* plants following infection with southern bean mosaic virus (SBMV), 10 acidic and 8 basic PR proteins have been identified, which include 4, 17 kDa serologically related, acidic proteins of unknown functions; 2 chitinases, 1 acidic (29 kDa) and 1 basic (32 kDa) possessing antifungal activities; and 4 (21, 28, 29, and 36 kDa) serologically related acidic glucanases (Mohamed and Sehgal, 1997).

Different isoforms of  $\beta$ -1,3-glucanases have been characterized in the infected tissues. Infection of groundnut leaves with the early leaf spot pathogen, *Cercospora arachidicola*, leads to a marked increase in the extracellular  $\beta$ -1,3-glucanase activity with the synthesis of its three isoforms (Roulin and Buchala, 1995). In pepper (*Capsicum annuum*) plants, it has been suggested that the glucanase activity is involved in the mechanism of resistance to cucumber mosaic virus in tolerant cultivars (Egea et al., 1996). TLPs are one group (PR-5, permatins) of antifungal PR proteins isolated from various plants. Transgenic tobacco plants constitutively overexpressing a rice TLP (PR-5) showed an enhanced resistance to *Alternaria alternata* (Velazhahan and Muthukrishnan, 2003).

Some of the PR proteins are present in healthy tissues and are differentially expressed by signals involved in flowering and reproduction. This implies that they are involved in the normal physiological processes of the plants in addition to plant defense (Samac and Shah, 1991). According to certain investigators, salicylic acid is involved in the signal transduction pathway, leading to a resistance to pathogen infection and the synthesis of PR proteins (Ferullo et al., 1997). The salicylic acid level increases in plants following pathogen attack (Samac and Shah, 1991). In barley leaves, a salicylic acid treatment induces the accumulation of two PR proteins and one salicylic acid-specific protein (Tamas and Huttova, 1996). In tobacco plants, salicylic acid acts as an endogenous signal for the expression of acidic PR-1 proteins (Conrath et al., 1997). However, using transgenic tobacco plants accumulating high levels of soluble sugars due to the cytosolic expression of an inorganic pyrophosphatase from *Escherichia coli*, the possible role of soluble sugars in the induction of PR proteins has been suggested (Badur et al., 1994; Herbers et al., 1996). Such an induction appeared to be salicylic acid independent in the source leaves of tobacco plants (Herbers et al., 1996). According to Malamy et al. (1996), multiple pathways exist that lead to defense response in plants, one of which appears to be independent of salicylic acid. More evidence is required to address the signal transduction pathways leading to the synthesis of PR proteins and the function of PR proteins in plant defense.

A stress-induced protein with an estimated molecular mass of 17 kDa and designated as FISP17 was identified using SDS-PAGE. The western blot analysis showed that the antibody only reacted with a 17 kDa protein in plant exudates infected with *Fusarium solani* f. Sp. *glycines*, but no reaction occurred with healthy plant exudates or with culture filtrates of *F. solani* f. Sp. *glycines* (Li et al., 2000). Comparative two-dimensional gel analyses revealed 21 differentially expressed protein spots in secreted proteins due to *Magnaporthe grisea* and/or elicitor in suspension-cultured rice cells over control. MALDI-TOF-MS and muLC-ESI-MS/MS analyses of these protein spots revealed that most of these assigned proteins were involved in defense, such as nine chitinases, two germin A/oxalate oxidases, five domain unknown function 26 (DUF 26) secretory proteins, and beta-expansin (Kim et al., 2009).

Microbe or elicitor-induced signal transduction pathways lead to an increased production of ROS and reactive nitrogen species (RNS). Among the proteins induced during the host plant defence, class III plant peroxidases are well known (Almagro et al., 2009). They belong to a large multigene family, and participate in a broad range of physiological processes, such as lignin and suberin formation, cross-linking of cell wall components, and synthesis of phytoalexins, or participate in the metabolism of ROS and RNS, both switching on the hypersensitive response, a form of programmed host cell death at the infection site associated with limited pathogen development (Almagro et al., 2009).

### 19.2.7 WOUNDING

Wounded plants manifest increases in the activities of many enzymes and the levels of proteins (Mehta et al., 1991; Cabello et al., 1994; Schaller and Ryan, 1996; Chen et al., 2005; Dafoe et al., 2009; Turrà et al., 2009). Enzymes and proteins that show an increased level in response to wounding include enzymes of the phenylpropanoid pathway, peroxidase, dihydroxyacetonephosphate synthase (DHAP synthase), glycine-rich and hydroxy-proline-rich cell wall proteins, protease inhibitors, and 1-aminocyclopropane-1-carboxylate synthase (Davis et al., 1990; Mehta et al., 1991). It is believed that some of these enzymes and proteins are involved in the lignification process and, thus, form a wound periderm to limit a pathogenic attack (Davis et al., 1990). Both glucanase and chitinase activities are induced in the roots and stems of chickpea (*C. arietinum* L.) plants in response to wounding (Cabello et al., 1994).

In tobacco crown gall tumor tissues, a 16 kDa glycine-rich hydrophobic polypeptide has been characterized, which is a cell wall protein and is induced by mechanical wounding (Yasuda et al., 1996). This polypeptide is involved in the wound-healing process in tobacco plants by modifying the cell wall composition (Yasuda et al., 1996). In tomato plants, several systemic wound-response proteins (swarps) have been described (Mehta et al., 1991; Schaller and Ryan, 1996). Mehta et al. (1991) noticed the appearance of several novel proteins of 80.0, 63.0, 33.0, 29.0, 28.5, and 25.5 kDa and a decrease in the level of a 15 kDa protein as a result of wounding in tomato fruit tissues. These investigators noticed a marked difference in the mRNA populations after wounding. A full-length cDNA encoding an aspartic protease (LeAspP) was cloned from a tomato leaf cDNA library, the mRNA of which was shown to be systemically induced by wounding (Schaller and Ryan, 1996).

One day after the leaves of sweet potato were wounded, the appearance of ipomoelin (IPO) protein was observed, which was found to be a lectin, a carbohydrate-binding protein (Chen et al., 2005). The genotype-dependent expression of specific members of potato protease inhibitor gene families was observed in different tissues in response to wounding (Turrà et al., 2009). The accumulation of a Kunitz trypsin inhibitor from chickpea (TPI-2) located in cell walls was reported to increase in wounded leaves (Jimenez et al., 2008). Shen and coworkers (2003) identified 10 upregulated proteins and 19 downregulated proteins in response to wounding in rice leaf sheath. Among all these proteins, the Bowman-Birk trypsin inhibitor, a putative receptor-like protein kinase, and a calmodulin-related protein have been confirmed to be wound-response proteins. Using comparative two-dimensional electrophoresis, it was observed that in poplar phloem

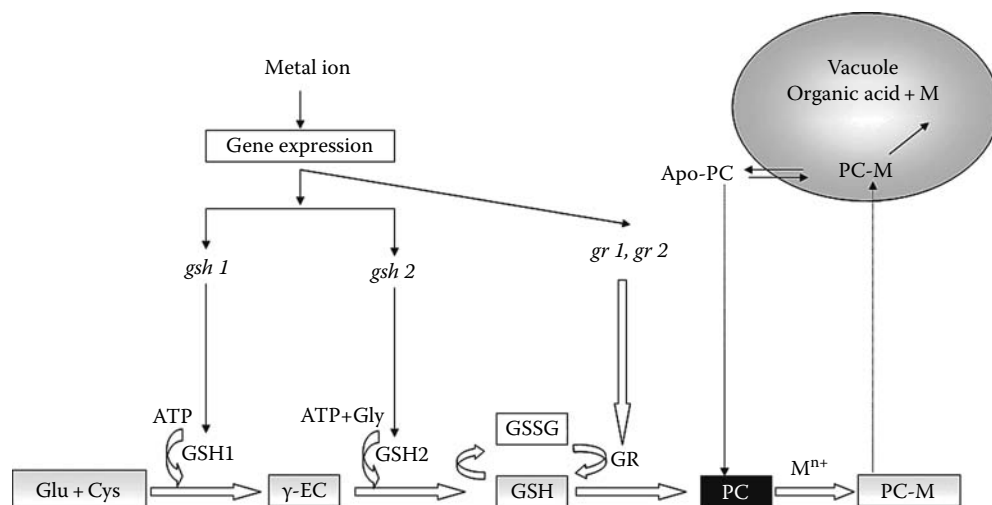
exudate, two proteins, pop3 and a TLP, were upregulated after 24 h of wounding (Dafoe et al., 2009). These observations suggest that wounding stress leads to a differential protein accumulation and an altered gene expression in tissues.

### 19.2.8 METAL TOXICITY

Industrialization has led to the increased introduction of several metals like Cd, Pb, Zn, Cu, and Hg in the soil environment. High levels of heavy metals in the soil adversely affect plant growth, cause the induction or inhibition of enzymes, and induce the synthesis of metal-binding cysteine-rich polypeptides called phytochelatins (PCs). PCs have a primary structure of  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$  or  $(\gamma\text{-Glu-Cys})_n\text{-Ala}$ , when  $n = 2\text{--}11$ , and have an apparent function in the sequestration of metal ions within the plant.

Cadmium, which is a major environmental pollutant, inhibits the activities of many enzymes owing to its interaction with  $-\text{SH}$  groups of enzymes (Shah and Dubey, 1995a,b). When grown in the presence of Cd, Cu, or As, many plants synthesize PCs. It is believed that PCs are synthesized from glutathione by the transpeptidation of  $\gamma$ -glutamyl cysteinyl dipeptides by the action of a constitutively present enzyme phytochelatin synthase (PC synthase) (Chen et al., 1997). PC binds to metal ions leading to the sequestration of metal ions in plants, and thus serves as an important component of the detoxification mechanism in plants. Apo-phytochelatin (non-metallated PCs) may get degraded by vacuolar hydrolases, and, in turn, PCs may return to cytosol where they could continue to carry out their shuttle role (Figure 19.6).

Partially purified PC synthase was found to be active only in the presence of metal ions. The best activator observed was Cd, followed by the cations Ag, Bi, Pb, Zn, Cu, Hg, and Au. These metals also induce PC biosynthesis in plant cell cultures under *in vivo* conditions (Grill et al., 1989). Similar PC synthase activities have been detected in plants such as pea (Klapheck et al., 1995), tomato (Chen et al., 1997), and *Arabidopsis* (Howden et al., 1995). In cell suspension cultures of red spruce (*Picea rubens* Sarg.), the PC and its precursor,  $\gamma$ -glutamylcysteine ( $\gamma\text{-EC}$ ), increased two- to fourfold with Cd concentrations ranging from 12.5 to 200  $\mu\text{M}$  as compared to the control. However, Zn-treated cells showed a less than twofold increase in  $\gamma\text{-EC}$  and PC levels as compared to



**FIGURE 19.6** Mechanisms involved in PC chelation and compartmentalization of metal ions in vacuole. Apo-phytochelatin (non-metallated PCs) may get degraded by vacuolar hydrolases and, in turn, PCs may return to cytosol, where they could continue to carry out their shuttle role. The synthesis of PCs is accompanied with a decrease in cell glutathione pool and increase in the activities of gamma-glutamyl-cysteine synthetase (GSH 1), glutathione synthetase (GSH 2), as well as GR. The elevated activities of GSH 1, GSH 2, and GR are correlated with the enhanced expression of corresponding genes *gsh 1*, *gsh 2*, *gr 1*, and *gr 2*.

the control even at the highest concentration of 800  $\mu\text{M}$ . In addition, unidentified higher-chain PCs were also found in both the Cd- and Zn-treated cells, and they increased significantly with increasing concentrations of Cd and Zn (Thangavel et al., 2007). Compared to wild-type plants, transgenic Indian mustard (*Brassica juncea* L.) plants overexpressing *A. thaliana*, *AtPCSI*, gene encoding PC synthase, exhibit significantly higher tolerance to Cd and As (Gasic and Korban, 2007). The overexpression of *AtPCSI* also resulted in an enhanced  $\text{Cd}^{2+}$  tolerance in the non-accumulator plant *N. tabacum* (Pomponi et al., 2006).

The synthesis of certain novel proteins with molecular weights greater than 14 kDa occurs when plants are exposed to cadmium (Choi et al., 1995; Shah and Dubey, 1998a). A Cd-binding protein complex with an apparent molecular weight of 18 kDa was purified by Shah and Dubey (1998a) from rice plants. This complex had a specific Cd content of 3.7  $\mu\text{moles mg}^{-1}$  peptide and 4 –SH groups per protein molecule. It is suggested that these protein complexes bind Cd with the help of –SH groups of the peptide in mercaptide bonds and help in the sequestration of excess Cd ions in plants (Shah and Dubey, 1998a). The isolation of a cDNA was reported by Choi and coworkers (1995), which was differentially expressed by 150 mM Cd in *Arabidopsis* plants and encoded an 18.3 kDa protein.

Lupin roots exposed to lead, copper, or nitrite ions showed an increased synthesis of a 16 kDa polypeptide, which appeared to be a cytosolic Cu:Zn-SOD (Przymusiński et al., 1995). From mercuric chloride-treated maize leaves, transcriptionally activated cDNA clones were isolated, which represented various known proteins, such as glycine-rich proteins, PR proteins, chaperons, and membrane proteins (Didierjean et al., 1996). A novel metallothionein (MT)-like protein, the expression of which is regulated by metal ions, osmoticum or ABA, has been isolated from Douglas fir trees during embryogenesis (Chatthai et al., 1997). These observations suggest that some of the heavy metal-induced proteins are also induced by other abiotic stresses.

MTs are low-molecular-weight (6–7 kDa), 60–65 amino acid residue long, cysteine (20 molecules)-rich metal-binding (through mercaptide bonds) proteins (Liu et al., 2000). These could also function as antioxidants, although evidence is lacking (Dietz et al., 1999), while a role in PM repair is another possibility (Salt et al., 1998). Based on the number and arrangement of cysteine residues, all plant MTs belong to class II (in contrast to the vertebrate class I), and can be further subdivided into four types based on their amino acid sequences (Robinson et al., 1993; Cobbett and Goldsbrough, 2002). Type-1 MTs function under various abiotic stresses, including exposure to the cadmium ion. *Brassica rapa* type-1 MT gene (*BrMT1*) has been found to confer resistance to Cd in otherwise Cd-sensitive yeast. The overexpression of this gene in either the chloroplasts or the cytosol effectively detoxifies cadmium in transgenic *Arabidopsis* (Kim et al., 2007). Plant type-2 MT *BjMT2* from *B. juncea* overexpressed in *A. thaliana* exhibited an increased tolerance against  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$  (An et al., 2006).

In rice seeds germinating in the presence of 200  $\mu\text{M}$  Cu for 6 days, 13 upregulated proteins were identified, including an MT-like protein, a membrane-associated protein, a putative wall-associated protein kinase, PR proteins, and the small putative GTP-binding protein Rab2 (Zhang et al., 2009). *A. thaliana* plants treated with 10  $\mu\text{M}$   $\text{Cd}^{2+}$  for 24 h showed an increased synthesis of PCs and 41 spots indicated significant changes in protein abundance. Most of the identified proteins belonged to four different classes: metabolic enzymes, such as ATP sulphurylase, glycine hydroxymethyltransferase, and trehalose-6-phosphate phosphatase; glutathione *S*-transferases; latex allergen-like proteins; and unknown proteins (Roth et al., 2006). The soybean cell suspension culture treated with various concentrations of  $\text{Cd}^{2+}$  showed a Cd-induced appearance of the SOD, histone H2B, chalcone synthase, and glutathione transferase proteins (Sobkowiak and Deckert, 2006).

Excess copper treatment resulted in an increased expression of three proteins in 10-day-old *P. vulgaris* roots. The levels of an intracellular PR protein and a newly identified protein homologous to PvPR1, PvPR2, were increased with increasing Cu concentration. At 50  $\mu\text{M}$  Cu exposure, the appearance of PvPR1 and a homologue of *A. thaliana* thylakoid luminal 17.4 kDa protein were observed in leaves. Another protein that was slightly enhanced by Cu treatment had a sequence homology to a mitochondrial precursor of the glycine cleavage system H protein of *Flaveria pringlei* (Cuypers et al., 2005). Limited evidences suggest that an increased synthesis of some HSPs

occurs in plants in response to metal stress, but the specific functions or structures protected by HSPs under metal stress remain unidentified (Heckathorn et al., 2004). Cd exposure has been shown to induce the synthesis of a considerable number of stress proteins, presumably HSPs, with the molecular weight ranging between 10 and 70 kDa (Sanita di Toppi and Gabbrielli, 1999). In cell cultures of *Lycopersicon peruvianum*, exposed to 1 mM Cd, significant amounts of HSP 70 appeared bound to plasmalemma, mitochondrial membranes, and endoplasmic reticulum (Neumann et al., 1994). HSP 70 has a strong affinity for misfolded proteins and helps them to find their native conformation by reintegrating them into the proper membrane complex (Sanita di Toppi and Gabbrielli, 1999). In *Z. mays* plants, Cu, Ni, Pb, and Zn led to a decrease in the net photosynthesis ( $Ph_n$ ) and an increase in the levels of chloroplast small HSPs (smHSPs) content, which increased with the time of exposure (Heckathorn et al., 2004). Under *in vitro* conditions, it was shown that the photosynthetic electron transport ( $Ph_e$ ) was protected from Pb (but not by Ni) by the addition of purified chloroplast smHSPs to the thylakoids. Under *in vivo* conditions,  $Ph_n$  was protected from Ni and Pb in the presence of increased levels of smHSPs in a heat-tolerant *Agrostis stolonifera* genotype expressing additional chloroplast smHSPs compared to a near-isogenic heat-sensitive genotype (Heckathorn et al., 2004). These results suggest that HSPs protect photosynthesis from metals and are among the first to demonstrate that specific functions are protected by HSPs during metal stress (Heckathorn et al., 2004).

#### 19.2.8.1 Enzyme Levels in Metal-Stressed Plants

Germinating seeds or plants growing under excessive metal levels show alterations in levels and activity behaviors of many enzymes. A suppression of proteolytic activity marked by decreased activities of protease and peptidase was observed due to Cd treatments in germinating rice seeds, leading to altered levels of protein and amino acids (Shah and Dubey, 1998b). An increase in the level of proteins and the increased activity of carboxypeptidase accompanied with a decline in the activity of protease and leucine aminopeptidase (LAP) and the level of free amino acid pool were observed in rice seedlings under arsenic supplementation compared to controls (Mishra and Dubey, 2006). Maheshwari and Dubey (2007) suggested that nickel toxicity in rice seedlings suppresses the hydrolysis of proteins and RNA by inhibiting the activity of protease and ribonuclease, respectively. Shah and Dubey (1995b) observed an increase in the ribonuclease activity in rice seedlings due to a moderate Cd treatment level of 100  $\mu$ M, whereas a higher Cd level of 500  $\mu$ M was inhibitory to the enzyme. Similarly, rice plants grown in the presence of 25 or 50  $\mu$ M arsenic showed a marked decline in the ribonuclease activity (Mishra and Dubey, 2006). A tissue-specific inhibition of the activities of phosphatases both under *in situ* and *in vitro* conditions has been observed due to Cd and As in growing rice plants (Shah and Dubey, 1998c; Mishra and Dubey, 2008b). Activities of the phosphorylolytic enzymes acid phosphatase, alkaline phosphatase, and inorganic pyrophosphatase decreased in rice plants grown under high Cd and As levels (Shah and Dubey, 1998c; Mishra and Dubey, 2008b).

The effect of increasing concentrations of Cd, As, and Al *in situ* on the enzymes related to sugar and starch metabolism were studied in rice seedlings (Verma and Dubey, 2001; Jha and Dubey, 2004a; Mishra and Dubey, 2008a). The activities of the enzymes of starch hydrolysis  $\alpha$ -amylase and  $\beta$ -amylase declined, whereas the activities of sucrose-hydrolyzing enzymes SS and acid invertase increased in the rice seedlings when grown in the presence of these metals, whereas the enzyme of sucrose synthesis, SPS, showed a decreased activity in Cd-, As-, as well as Al-treated seedlings compared to controls (Verma and Dubey, 2001; Jha and Dubey, 2004a; Mishra and Dubey, 2008a). An enhanced activity of a starch phosphorylase enzyme was observed in As-stressed rice seedlings (Jha and Dubey, 2004a). Devi and coworkers (2007) also observed marked changes in the activity of enzymes of carbohydrate metabolism, glycolysis, and the pentose phosphate pathway in pea plants under cadmium toxicity. They suggested that higher activities of hexokinase and phosphoglucosomerase in the roots and shoots of cadmium-stressed seedlings might be due to preferable channeling of hexose toward glycolysis than toward the pentose phosphate pathway. A marked inhibition in the activities of the nitrate assimilatory enzymes NR, nitrite reductase (NiR), and GS is observed in



rice seedlings subjected to arsenic toxicity, whereas elevated activities of AlaATs and AspATs are observed (Jha and Dubey, 2004b). Aluminum toxicity is also reported to decrease the total amount of functional NR in rice seedlings (Sharma and Dubey, 2005b). Rice seedlings subjected to 80  $\mu\text{M}$   $\text{Al}^{3+}$  showed a decreased level of NR max (NR activity in the presence of EDTA), but resulted in higher NRact (NR activity in the presence of  $\text{Mg}^{2+}$  representing the non-phosphorylated NR state) and NR activation state. However, seedlings grown in the presence of a higher level of 160  $\mu\text{M}$   $\text{Al}^{3+}$  showed a decline in NRact as well as in NRmax.

Metals like Cd, Al, Pb, and Ni have been shown to induce the activity of antioxidative enzymes, namely, guaiacol peroxidase (GPX), SOD, APX, and GR, and enzymes of the ascorbate-glutathione cycle (MDHAR, DHAR, and GR) (Shah et al., 2001; Verma and Dubey, 2003; Qureshi et al., 2007; Sharma and Dubey, 2007; Maheshwari and Dubey, 2009). However, Schutzendubel and coworkers (2001) showed that metals like Cd can decrease some of the antioxidant enzymes, leading to the accumulation of  $\text{H}_2\text{O}_2$ , and hence such plants would suffer from oxidative stress. Ezaki and coworker (2001) observed the  $\text{Al}^{3+}$ -led induction of tobacco glutathione *S*-transferase gene (*parB*) and tobacco peroxidase gene (*NtPox*). A highly toxic Pb (1000  $\mu\text{M}$ ) level has been shown to decrease the intensity of two preexisting catalase isoforms in shoots of rice seedlings (Verma and Dubey, 2003). Sharma and Dubey (2007) observed the appearance of two new isoenzymes of APX in the roots of Al-treated seedlings compared to control. Using the western blot analysis, Sharma and Dubey (2007) showed that changes in the activities of APX due to  $\text{Al}^{3+}$  toxicity are due to changes in the amounts of enzyme protein. However, the activation of antioxidant enzymes in response to oxidative stress induced by metals is generally reported to be not sufficient enough to confer tolerance to metal accumulation.

### 19.2.9 GASEOUS POLLUTANTS

Ozone ( $\text{O}_3$ ), sulfur oxide ( $\text{SO}_2$ ), and nitric oxide ( $\text{NO}_2$ ) are considered as the major air pollutants. These molecules generate ROS, inhibit the synthesis of many proteins, and induce the activities of some antioxidant enzymes (Conklin and Last, 1995; Glick et al., 1995; Carreras et al., 1996; Rao et al., 1996; Sharma and Davis, 1997; Agrawal et al., 2002; Torres et al., 2007). Plants grown in places with high industrial pollution levels exhibit significantly low concentrations of soluble proteins (Carreras et al., 1996).

Ozone fumigation causes a decrease in the steady-state mRNA levels of genes encoding the small subunit of RUBISCO, a chlorophyll a/b-binding protein, and a 10 kDa protein of the water-evolving complex of PSII (Conklin and Last, 1995). Similarly, in potato plants, ozone accelerates senescence with a decline in the small-subunit RUBISCO mRNA as well as a decline in the transcripts of glyceraldehyde-3-phosphate dehydrogenase (Glick et al., 1995). Agrawal et al. (2002) reported the appearance of new phosphoproteins in ozone-treated rice leaves. Furthermore, a 66 kDa protein in leaf extracts showed a strong and specific cross-reaction with an anti-MAPKinase (ERK1) antibody, whose levels increased within 5 min of ozone exposure, suggesting possible involvement of ERK-type MAPKs in the ozone-elicited self-defense response pathway(s) in rice.

Many antioxidant enzymes show an increased level when plants are exposed to ozone. Cytosolic Cu/Zn-SOD is the best characterized enzyme, which shows an increased activity with the simultaneous synthesis of new isoforms, in plants subjected to  $\text{O}_3$  exposure (Conklin and Last, 1995; Rao et al., 1996). In *A. thaliana*,  $\text{O}_3$  exposure enhances the activities of SOD, peroxidase, GR, and ascorbate reductase, and modifies the substrate affinity of both GR and APX (Conklin and Last, 1995; Rao et al., 1996). However, in the chloroplasts of ozone-exposed plants, a decline in the levels of Fe-SOD and GR has been observed (Conklin and Last, 1995). An ozone-induced transcript has been characterized in *A. thaliana* that encodes an 8.6 kDa basic protein, which represents a novel stress-related protein (Sharma and Davis, 1997). It is suggested that the major classes of ozone-induced proteins include antioxidant enzymes and a number of stress-related proteins associated with other biotic and abiotic stresses, and that ozone-induced responses are caused in part by the activation of

a salicylic acid-dependent signaling pathway (Sharma and Davis, 1997). When the responses of cultivated bean (*P. vulgaris* L. cv. IDIAP R-3) and maize (*Z. mays* L. cv. Guarare 8128) plants exposed to ozone were studied using a proteomics approach, changes in the RUBISCO protein levels were observed (Torres et al., 2007). In bean leaves, two SOD proteins (19 and 20 kDa) decreased dramatically, while APX (25 kDa), a small HSP (33 kDa), and a naringenin-7-O-methyltransferase (NOMT, 42 kDa) increased under O<sub>3</sub> exposure. In maize leaves, the expression levels of catalase (increased), SOD (decreased), and APX (increased) changed greatly by O<sub>3</sub> depending on the leaf stage, whereas crossreacting HSPs (24 and 30 kDa) and NOMT (41 kDa) proteins strongly increased in O<sub>3</sub>-stressed younger leaves. These results indicated a clear modulation of oxidative stress-related, heat shock-related, and secondary metabolism-related proteins by O<sub>3</sub>. A novel PR protein 2 was suggested as a potential marker for O<sub>3</sub> stress in bean (Torres et al., 2007).

### 19.2.10 UV RADIATION

Owing to the depletion of stratospheric ozone, in the future, the influx of solar UV-B radiation (280–320 nm) will tend to increase. UV-B radiation inhibits the growth of plants, causes inhibition in protein synthesis (Strid et al., 1994), and induces the activities of peroxidase-related enzymes (Rao et al., 1996) and the enzymes of the flavonoid biosynthetic pathway (Kuhn et al., 1984). Leaf protein biosynthesis is rapidly inhibited by UV-B radiation (Kuhn et al., 1984).

The chloroplast appears to be the main target of UV-B radiation damage. Very early events of UV-B damage include the decrease of mRNA transcripts for the photosynthetic complexes and other chloroplast proteins (Strid et al., 1994). In pea leaves, exposure to UV-B radiation for 7 days causes a rapid inhibition in protein synthesis and a reduction in mRNA transcripts for the chlorophyll a/b-binding protein (Jordan et al., 1994). Genes encoding defense-related enzymes, for example, of the flavonoid biosynthetic pathway, are rapidly upregulated on UV-B exposure. The activity of flavonoid-synthesizing enzymes, especially phenylalanine ammonia-lyase, 4-coumarate:CoA ligase, chalcone synthase, and UDP-apiose synthase, are induced in a coordinate way with UV-B treatment (Kuhn et al., 1984). Peroxidase-related antioxidant enzymes are induced under UV exposure (Rao et al., 1996). In *A. thaliana*, UV-B exposure preferentially enhances GPX, APX, and peroxidase specific to coniferyl alcohol, and modifies the substrate affinity of APX (Rao et al., 1996). In certain plants, the synthesis of some novel proteins has been reported under UV-B irradiation (Gregersen et al., 1994; Jung et al., 1995; Didierjean et al., 1996). UV-B exposure in sunflower leaf disks causes the induction of PR3 and PR5 proteins (Jung et al., 1995), whereas the expression of a membrane channel protein and PR proteins is induced in maize (Didierjean et al., 1996). The accumulation of an atypical transcript encoding a 42.3 kDa polypeptide and showing sequence similarity to O-methyltransferase (OMTs) from different plant species has been observed in barley leaves after UV-B treatment (Gregersen et al., 1994). It is suggested that the response to increased levels of UV-B radiation is dependent on the developmental stage of the tissues and involves complex changes in gene expression (Jordan et al., 1994).

At an early stage of bean growth, UV-B radiation increases the synthetic rate of proteins in leaves, and at later growth stages, it decreases the synthetic rate of proteins and the content of soluble protein, and increases the activities of proteolytic enzymes and the content of total amino acids (Feng et al., 1999). The results of SDS-PAGE indicated that UV-B radiation increased the contents of polypeptides with mol. wt. of 99, 88, 76, 42, 35, and 33 kDa at the early stage, and increased those with mol.wt. of 10–14, 29, 33, and 35 kDa at the later growth stage. It is suggested that the increase of these polypeptides is an adaptation response of plants to the enhanced UV-B radiation (Feng et al., 1999).

Casati and coworkers (2005), while investigating the effects of UV-B on the proteome of maize leaf, observed that UV-B radiation regulated the appearance of 178 protein spots and phosphorylated pyruvate phosphate dikinase. An immunoblotting analysis with an anti-AOX (alternative oxidase) monoclonal antibody revealed that the expression of the AOX protein increased in red kidney bean leaves under enhanced UV-B radiation (Zhao et al., 2007). Stilbene synthase (STS) is a pivotal

enzyme that catalyzes the biosynthesis of resveratrol and is known to be induced by UV irradiation. Pan and coworkers (2009) reported the increased activity of STS on the cell wall and suggested that it might be related to grape berry defense against UV irradiation.

### 19.3 CONCLUSIONS

Environmental stresses like salinity, drought, heat, chilling, anaerobiosis, heavy metals, gaseous pollutants, and UV radiation cause an alteration in the gene expression of plants, leading to the induction of specific genes and an increased abundance of their translatable mRNAs and proteins. As a result, an increased synthesis of certain novel proteins occurs in stressed plants with a concomitant decrease in the level of certain preexisting proteins. These stress-specific proteins appear to endow plants with the capacity to adapt to a stressful environment through physiological and biochemical adjustments. Most of these stresses induce the synthesis of proteins specific to the particular stress. However, certain proteins are common and can be synthesized under more than one type of stress. For example, cold, drought, and salinity stresses cause the expression of some common proteins that can also be induced by the treatment of normal tissues with ABA. Many of the genes that are activated under stressed conditions have been isolated and sequenced. Most of the stress-induced proteins have been isolated and well characterized for their physicochemical properties, and their amino acid sequences have been determined.

Although stress-specific proteins are thought to lend a protective role to the tissues against the deleterious effects of stress, the exact physiological functions of many of these proteins are not very clear. Further experiments are necessary to determine the functions of these proteins. Proteins induced under salinity or drought are believed to act as osmoprotectants, as regulatory proteins, or as enzymes of biosynthetic pathways for osmolytes. However, the functions of many proteins are still unknown. The HSPs are believed to perform many essential functions in normal as well as stressed cells. The molecular mechanisms underlying these processes and the role of HSPs in protecting heat-stressed tissues remain to be elucidated. Specific proteins are synthesized during cold treatments that improve freezing tolerance in crops, but the functions of these proteins in the acquisition of cold tolerance remain to be established. With the advent of the tools of genomics and proteomics, it has been possible to identify and characterize the whole set of stress-responsive genes, to determine their expression patterns under stresses, and to identify and characterize the entire spectrum of proteins that are overexpressed and those that are repressed under stresses. The identification of novel stress-responsive proteins and others, especially the proteins associated with stress tolerance, provides new insights into our understanding of the mechanisms of stress responses and tolerance in plants. Functional analyses of these proteins will be of immense help in producing stress-tolerant plants using transgenic approaches.

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# 20 Heat Shock Proteins and Acquisition of Thermotolerance in Plants

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## 20.1 INTRODUCTION

Plants growing in natural environments encounter various abiotic stresses during all parts of their growth and development. Abiotic stresses such as high temperature, low temperature, high light (photooxidative), drought, and salt stresses are the primary causes of crop yield and quality reductions. High temperature or heat stress can lead to retardation in growth and development, and even death. It causes protein dysfunction/damage and alters the cellular proteome. They can lead to accumulation of toxic protein aggregates. Plants as well as other organisms respond to heat stress by inducing heat shock proteins (Hsps) for self-defense. Hsps are ubiquitous, highly conserved proteins in living organisms that play a role for the maintenance of protein homeostasis in cells.

In this chapter, we summarize the recent progress made toward understanding the roles of Hsps in terms of thermotolerance mainly in plants. A number of excellent reviews have been published and the reader should also consult these publications for detailed information and discussions concerning plant Hsps [1–14].

## 20.2 HEAT SHOCK RESPONSE

Increase in temperature above the normal growth temperature of an organism induces a wide variety of perturbations in cellular structures and metabolic processes (see Ref. [14] and references therein). When the magnitude and duration of the heat stress exceeds a threshold, the cells are irreversibly damaged and they die. Plants that cannot move rely on proteomic plasticity to remodel themselves to survive under the stress. Proteome analysis of rice leaves showed that among 48 proteins differentially expressed upon heat shock at 42°C for 12 or 24 h, 18 up-regulated proteins were Hsps (small Hsp, Hsp60/Cpn60, Hsp70, and Hsp90) [15]. Other differentially expressed proteins were categorized into classes related to energy and metabolism, redox homeostasis, and regulatory proteins.

In general, the induction of Hsps is rapid, intense, and transient, suggesting that it is an important emergency response. Transcripts of Hsps can be detected as early as 5 min after heat shock (for example, see Ref. [16]). Their levels may reach peak within 30 min of heat shock exposure.

In eukaryotic cells, the heat shock response involves transcriptional activation mediated by heat shock factors (Hsfs). They recognize palindromic binding motifs—heat shock elements conserved in promoters of heat-shock-inducible genes of all eukaryotes (see Ref. [17] and references therein). Compared with other eukaryotes with one to three Hsfs, the plant Hsf family shows a striking multiplicity, with more than 20 members. Among them, HsfA2 is the dominant Hsf in thermotolerant cells [18], although other Hsfs also contribute for cellular thermotolerance as described below.

## 20.3 FIVE REPRESENTATIVE HSP FAMILIES

Five Hsp families are recognized as highly conserved among different species and classified on the basis of their approximate molecular masses [1–3,6,14]: Hsp100/ClpB, Hsp90/HtpG, Hsp70/DnaK, chaperonin (Hsp60)/GroEL, and small HSP (also called or abbreviated as low molecular weight/mass Hsp, small stress protein, sHsp, or  $\alpha$ -Hsp).

A single plant species has multiple members for an Hsp family. Some members of an Hsp family are heat-induced. In the above mentioned proteome analysis [15], 3 members of the Hsp100, 7 of Hsp70, and 7 of small Hsp families were heat-induced. Some members of an Hsp family may be induced by physical or chemical stresses as described later. These suggest that some Hsp members may have evolved to acquire specialized function(s) in response to specific type(s) of stresses to cope with various hostile environments.

## 20.4 IMPORTANCE OF HSPS UNDER STRESS CONDITIONS AS WELL AS NORMAL CONDITIONS

The importance of the Hsp induction in cellular thermotolerance comes from the apparent correlation between the level of Hsp accumulation and thermotolerance. When whole organisms or cultured cells are given short treatments at moderately elevated temperature, their resistance to extreme heat increases dramatically [19]. For example, when yeast cells grown at 25°C are pretreated at 37°C, they can survive at 50°C 1,000 fold better than non-pretreated cells. This increase in thermotolerance is observed in virtually every organism studied [19]. Such tolerance inducing treatments generally also induce the synthesis of Hsps. The extent of the Hsp induction appears to be correlated with the dose of heat stress that an organism is subjected to.

Besides a short and rapid increase in temperature, a treatment that has often been used for an experiment in a laboratory, but may not have relevance to plants in the field, a gradual increase in temperature also confers thermotolerance in plants [20,21]. This gradual increase in temperature effectively elicited the synthesis of Hsps as well. While identifying important molecular events for acquired thermotolerance, *Arabidopsis thaliana* was treated to severe heat stress (45°C) without acclimation or following two different acclimation treatments. Notably, a gradual increase to 45°C (22°C to

45°C over 6h) led to higher survival and higher-fold transcript changes than a stepwise acclimation (90min at 38°C plus 120min at 22°C before 45°C). A significant difference in the total spectrum of transcript changes in the two treatments was observed, although core components of heat acclimation overlapped between treatments. Finally, eight new genes involved in heat acclimation were defined by T-DNA insertion mutants, and one of them was the transcription factor HsfA7a [22].

Importance of Hsps extends beyond their potential role in protection from high temperature and other stresses. Although Hsps are induced by different stresses, some Hsps are also expressed under unstressed conditions. For example, one of the members of the *Arabidopsis* Hsp90 family is constitutively expressed and its expression is slightly enhanced by elevated temperatures, whereas another one that stays in a very low level at normal temperatures is strongly induced by heat [23]. Some Hsps are produced at particular stages of the cell cycle or during development in the absence of stress [1,3,14].

An accumulated body of evidence has indicated that all the five representative Hsps function as molecular chaperones as described below, and are involved in general and essential cellular functions; for example, protein folding and subunit assembly, protein translocation across membranes, signal transduction, and intracellular protein breakdown [24]. In fact, some Hsps such as *Escherichia coli* GroEL (Hsp60), and yeast Hsp82 (Hsp90) were shown to be essential for viability [25,26]. Not all but one or two members of the multiple DnaKs that are present in a cyanobacterial cell are essential [27,28]. Similar situation is also found in the case of cyanobacterial DnaJ (Hsp40), an essential component of the DnaK chaperone system (see below section) [29]. Cyanobacteria are thought to be ancestors of chloroplasts. Thus, The Hsp70/DnaK chaperone system may play an essential role for chloroplast function.

## 20.5 HSPTS AS MOLECULAR CHAPERONES

The major Hsps are molecular chaperones. Laskey et al. [30] originally used the term molecular chaperone in order to describe the properties of nucleoplasmin, an acidic nuclear protein necessary to correctly assemble nucleosome cores out of DNA (acidic) and histone (basic). Ellis [31] later defined the term as follows: Molecular chaperones are a class of unrelated families of proteins that have in common the ability to assist the non-covalent assembly of other protein-containing structures *in vivo*, but which are not permanent components of these structures. In this definition “assembly” is used in a broad sense to include not only the folding of newly synthesized polypeptide chains and any association into oligomers that may happen subsequently, but also any changes that may occur when proteins are translocated across membranes, perform their normal functions, or are repaired/removed after being damaged by stresses.

### 20.5.1 MECHANISM FOR MOLECULAR CHAPERONES TO MAINTAIN PROTEIN HOMEOSTASIS

Temperature increase can lead to denaturation of a protein or a protein complex, resulting in the exposure of hydrophobic amino acid residues normally buried within the interior of the protein [31,32]. A denatured protein may misfold and/or aggregate due to the interaction of the surface exposed by the hydrophobic region with other such regions of itself or other denatured proteins. Molecular chaperone binds to the exposed hydrophobic surface of a nonnative protein to prevent its misfolding and/or aggregation and subsequently facilitates refolding to its native conformation and assembly into a protein complex. Molecular chaperones can prevent improper interactions among surface exposed hydrophobic regions of a protein(s). Their interaction with denatured proteins is transient and they are not present in native proteins/protein complexes. Thus, they behave like enzymes.

Presently, three enzyme-like activities are described for molecular chaperones. First, is the “foldase” activity that allows them to facilitate protein folding. Second, is the “holdase” activity by which they can bind an unfolding protein to prevent its aggregation and holds it in a folding/assembly competent state. Third, is the “disaggregase” activity that rescues proteins from an aggregated state. Not all Hsps have all of these activities. We discuss below the activities in each Hsp family.

### 20.5.2 SMALL Hsp

The small Hsps are found in eubacteria, eukaryotes, and archaea [33–35]. They are ubiquitous in terms of cellular localization as well as the biological world. Often, multiple members of the small Hsp family are present in one cellular compartment. Thus, the small Hsp family in plants is classified into cytosolic class I, cytosolic class II, cytosolic class III, chloroplast, mitochondrion, endoplasmic reticulum, and peroxisome small Hsps [1,2,4,36–40].

The small Hsps whose monomeric molecular masses range from 12–42 kDa are the most diverse in primary structure amongst the major Hsps. They are characterized by their secondary and tertiary structures. They have a core  $\alpha$ -crystallin domain of approximately 100 amino acids, which is flanked by an N-terminal arm and a short C-terminal extension. The  $\alpha$ -crystalline domain is built from an immunoglobulin fold that consists of a  $\beta$ -sandwich, comprising two anti-parallel  $\beta$ -sheets [41,42]. The majority of small Hsps form oligomers of 12 to >32 subunits.

Small Hsps are ATP-independent holdases. They bind nonnative proteins to form a soluble complex, preventing their aggregation. The N-terminal arm and/or C-terminal extension may mediate the binding to the substrate proteins. They may also be involved in the oligomer formation of small Hsps, and thus the dissociation of the small Hsp oligomers may be necessary to make the N-terminal arm and/or C-terminal extension available for interaction with nonnative proteins. The small Hsp/nonnative protein complex serves as a transient reservoir of substrates for subsequent refolding by ATP-dependent chaperone systems such as the Hsp70/DnaK chaperone system (see Ref. [35] and references therein). Small Hsps even coaggregate with nonnative proteins in order to mediate the resolubilization of the aggregates and subsequent refolding by ATP-dependent chaperone systems such as Hsp100/ClpB and the Hsp70/DnaK chaperone system (see Ref. [35] and references therein). One notable structural feature of chloroplast small Hsp (Hsp21) is that it contains a set of conserved methionines. Methionines M49, M52, M55, M59, M62, and M67 are located on one side of an amphipathic helix, which may fold back over two other conserved methionines (M97 and M101), to form a binding groove lined with methionines for the recognition of proteins with an overall hydrophobic character [43]. As small Hsps protect other proteins from aggregation by binding to their hydrophobic surfaces, keeping the conserved methionines in a reduced form is a prerequisite to maintain such binding.

Evidence for the involvement of small Hsps in plant thermotolerance is found in literature, for example, enhanced thermotolerance of transformed tobacco plants with the introduction of the tomato mitochondrial small Hsp gene [44], increased thermotolerance by the overexpression of a small Hsp (sHsp17.7) in rice [45], and increased thermotolerance observed in *Arabidopsis* that constitutively expresses a cytosolic class I small Hsp from *Rosa chinensis* [46]. Carrot transgenic cells and regenerated plants, which constitutively expressed the carrot small Hsp (Hsp17.7) gene showed more thermotolerance than the vector controls [47]. In contrast, heat-inducible Hsp17.7 antisense lines were less thermotolerant.

Small Hsp has not only the protein-protective activity, but also an ability to stabilize lipid membranes [48]. A mutant small Hsp with increased thylakoid association provided an elevated resistance against UV-B damage in the cyanobacterium *Synechocystis* sp. PCC 6803 [49]. In this cyanobacterium, it was shown that the wild type small Hsp (Hsp17) is equally distributed between the thylakoid and cytosolic fractions, whereas a mutant small Hsp17 mutated in position L9P appeared primarily in the soluble fraction, and another one mutated in position Q16R was found exclusively in the thylakoid membrane fraction. Compared with the wild type, the Q16R mutant had an enhanced lipid-mediated thylakoid membrane interaction, which affected directly the photosystem II complex and led to a greatly enhanced resistance to UV-induced photosystem II inactivation via facilitating photosystem II repair [49]. In this connection, it is interesting to note that immuno-cytochemical studies showed that cyanobacterial small Hsp has dynamic properties to change its localization between cytosol and thylakoid membranes during heat shock [50].

### 20.5.3 CHAPERONIN (Hsp60)/GroEL

Like small Hsps, chaperonins (Hsp60s)/GroELs are found throughout all three domains. They are ubiquitous in terms of cellular localization as well as the biological world, existing in cytosol, chloroplast, and mitochondrion. Chaperonins (Hsp60s)/GroELs (at least one of them when multiple homologues are present in a cell) are essential under all cellular conditions (for example, see Ref. [25]).

The two subfamilies of this Hsp family divide along recognizable evolutionary lines [51]. Type I is found in bacteria (GroEL) and endo-symbiotically related organelles, mitochondria (Hsp60), and chloroplasts (cpn60 or Rubisco binding protein), whereas type II resides in the archaeobacterial/eukaryotic cytosol (CCT/TriC). Type I and type II chaperonins are distantly related to each other.

Both type I and type II chaperonins are megadalton-size double-ring assemblies that are composed of approximately 60 kDa subunits. The assemblies provide an encapsulated cavity where a nonnative polypeptide folds productively. What differentiates type I and type II chaperonins structurally is the dependency on a detachable “lid” structure for encapsulation. Type I chaperonins require a co-chaperonin (cpn10, cpn20, Hsp10, or GroES) to close the cavity, while type II chaperonins have a built-in protrusion structure to perform this function.

Chaperonins are ATP-dependent foldases. They provide essential assistance to the folding/refolding of newly translated/nonnative and newly translocated proteins. Despite the different encapsulation mechanisms, the ATP-directed chaperone cycles of the two subfamilies appear to be similar [51]. We will discuss about type I chaperonins, GroEL, and chloroplast chaperonin below. We will not discuss about type II chaperonins since very little is known about the type II chaperonins and cytosol protein folding in plants.

#### 20.5.3.1 Type I Chaperonin

Among the type I chaperonins, the *E. coli* GroEL is most extensively studied. The chaperonin forms a large oligomer that is composed of fourteen 60 kDa GroEL [52,53]. The subunits are arranged in a barrel-like complex that is made up of two stacked heptameric rings, which enclose a “cavity.” GroEL consists of three domains: an equatorial domain that forms the foundation of the assembly, a loosely structured apical domain that forms the ends of the ring, and a slender intermediate domain that connects the two [53,54]. The equatorial domain includes most of the connections between monomers of the same ring and between rings and contains the ATP/ADP/Mg<sup>2+</sup>-binding pocket. The intermediate domain closes on the binding pocket, providing essential residues for ATP hydrolysis. The apical domain binds GroES or a substrate polypeptide. GroES, also called co-chaperonin, forms a single heptamer ring and plays an important role in the GroEL's chaperone action as described below.

How does GroEL function as an ATP-dependent foldase? The principal mechanism is summarized as follows [51]. An “open” ring with or without ATP may accept a nonnative polypeptide. Binding of ATP to the equatorial domains of the ring renders the apical domains competent to bind GroES, whose association is accompanied by a further large movement of the apical domains. This drives the release of polypeptide substrate protein from the cavity wall into the now encapsulated hydrophilic, so-called *cis* cavity, where folding then commences before ATP hydrolysis in the *cis* ring weakens the affinity of GroEL for GroES and leads the entry of ATP into the sites of the opposite, so-called *trans* ring. The binding of ATP triggers release of the *cis* ligands (GroES, the substrate protein, and ADP). At the same time, ATP binding in the *trans* ring also enables binding of GroES to that ring, triggering a further round of protein folding.

#### 20.5.3.2 Chloroplast Type I Chaperonin

Chloroplast chaperonin (cpn60) was first discovered as the Rubisco binding protein [55]. There are two types of cpn60,  $\alpha$ , and  $\beta$ , whose nuclear encoded precursors are synthesized outside the chloroplast and imported into it. The proteins are constitutively expressed and the level increases

only slightly during heat shock. The two chaperonins are only 50% identical in terms of amino acid sequences, and the occurrence of highly divergent cpn60s in a higher plant plastid appears to be general [56–58]. In spinach, these subunits can form hetero-oligomers as well as homo-oligomers [59]. To find out the significance of these isoforms, the  $\alpha$  and  $\beta$  subunits of cpn60 from pea were expressed individually in *E. coli*, and the purified cpn60s were subjected to *in vitro* reconstitution experiments [60]. In the presence of ATP, the  $\beta$  subunits formed homo-tetradecamers. In contrast,  $\alpha$  subunits only assembled into  $\alpha/\beta$  hetero-tetradecamers in the presence of  $\beta$  subunits. Nothing is known about the physiological significance for the presence of two types of chaperonins in chloroplasts. The following studies may provide a hint to solve the problem. In contrast with *E. coli*, cyanobacterial genomes generally contain two *groEL* homologues. The two GroELs are approximately 60% identical in terms of amino acid sequences. Although one of these GroELs is dispensable under normal growth conditions, it plays an important role(s) under stresses including heat and cold [61].

### 20.5.3.3 Chloroplast Co-Chaperonin

Chloroplast co-chaperonin was originally isolated as a protein from pea chloroplast lysate that formed a stable complex with GroEL in the presence of ATP [62]. Remarkably, it had twice the molecular mass of the GroES co-chaperonin. *In vitro* assay showed that it is a functional homolog of GroES. Genes encoding this “double” GroES-like co-chaperonin from spinach and *Arabidopsis* were cloned and the predicted amino acid sequences revealed that the co-chaperonin consists of two GroES-like sequences fused head-to-tail to form a single protein (see Ref. [63] and references therein). The two GroES-like sequences exhibit 40%–50% sequence identity. *In vivo*, the *Arabidopsis* homolog that was introduced and overexpressed in tobacco localized specifically to the chloroplast stroma [64].

Studies with purified proteins revealed a similar mechanism of protein folding in the case of the chloroplast chaperonin and co-chaperonin with that of GroEL and GroES [62,65,66]. Experiments showed that cpn60 functions equally well with bacterial, mitochondrial, or chloroplast co-chaperonin [60,66]. This means that the unique binary chloroplast protein is not obligatory for the cpn60-mediated folding. In addition to the cpn20, *Arabidopsis* chloroplast has a cpn10 that has only one GroES-like sequence [67].

Chloroplast cpn60 forms a stable complex with a variety of proteins besides Rubisco [68–71]. These are the Rieske FeS protein [69], ferredoxin NADP<sup>+</sup> reductase [70], and the multisubunit coupling factor CF<sub>1</sub> core complex [71].

### 20.5.4 Hsp70/DnaK

Hsp70 or DnaK (a prokaryotic homologue), is present in eukaryotic and eubacterial cells. Genes for Hsp70/DnaK homologues are only found in a subset of archaea [72]. Hsp70s that are highly conserved proteins [73] are present in cytoplasm, mitochondrion, chloroplast, endoplasmic reticulum, and nucleus [3,74–78]. Multiple members of the Hsp70 family can be present in one cellular compartment of higher plants. The Hsp70/DnaK family contains both heat-inducible and constitutively expressed members, the latter of which are sometimes called heat-shock cognate proteins (Hsc70). Many of higher plant Hsp70s show organ-specific expression pattern.

Hsp70/DnaK consists of an N-terminal ATPase domain of 45 kDa and a C-terminal peptide-binding domain of ~25 kDa [79,80]. The C-terminal domain is further subdivided into a  $\beta$ -sandwich subdomain (a substrate binding cavity) and an  $\alpha$ -helical subdomain. The latter is a lid that closes the cavity. Structures of the individual domains have been available [79,80], and recently a two-domain crystal structure of bovine Hsc70 was reported [81].

Hsp70/DnaK interacts with extended hydrophobic peptide segments in an ATP-controlled fashion [79,80]. ATP binding to the N-terminal ATPase domain triggers the transition to the low affinity state of the C-terminal peptide-binding domain, while ATP hydrolysis (to the ADP-bound state) leads to the high affinity state. The ATP binding opens the lid described above, which is

involved in substrate release. Substrate and/or Hsp40/DnaJ binding to Hsp70/DnaK stimulates ATP hydrolysis (see the following section). Thus, ATPase and substrate-binding domains mutually control each other allosterically.

Hsp70/DnaK plays diverse roles in cells, including (re)folding of newly synthesized or unfolded polypeptides, assisting in the import of proteins into organelles, the dissociation of macromolecular complexes/aggregates, and targeting proteins to lysosomes or proteasomes for degradation [79,80,82,83]. Hsp70/DnaK can be an ATP-independent holdase by itself, but becomes ATP-dependent foldase when it works with Hsp40/DnaJ and nucleotide exchange factor/GrpE. In a cell, Hsp70/DnaK works with these co-chaperones.

Transgenic *Arabidopsis* whose level of Hsp70/Hsc70 was reduced by an Hsp70 antisense gene exhibited less thermotolerance than the wild type [84]. Mutational studies indicated that a chloroplast member plays a role for thermotolerance. Two plastid Hsc70 (cpHsc70-1 and cpHsc70-2) T-DNA insertion knockout mutants,  $\Delta$ cphsc70-1 and  $\Delta$ cphsc70-2, of *Arabidopsis* were isolated. Although no visible phenotype was observed in the  $\Delta$ cphsc70-2 mutant under normal growth conditions, the  $\Delta$ cphsc70-1 mutant plants exhibited variegated cotyledons, malformed leaves, growth retardation, and impaired root growth. After heat shock treatment of germinating seeds, root growth from  $\Delta$ cphsc70-1 seeds was further impaired, indicating that cpHsc70-1 is important for thermotolerance of germinating seeds [85]. Transgenic tobacco plants that constitutively expressed elevated levels of nucleus-localized Hsp70 (NtHSP70-1) showed higher levels of thermotolerance than antisense transgenic seedlings or transgenic seedlings carrying only the vector [78].

#### 20.5.4.1 The Hsp70/DnaK Chaperone System

Hsp40/DnaJ (or J-domain proteins) is present in cytoplasm, mitochondrion, chloroplast, and endoplasmic reticulum [86–90]. The coexistence of Hsp70/DnaK and Hsp40/DnaJ in the same cellular compartment suggests that certain chaperone–co-chaperone interactions are permitted. Hsp40s/DnaJs are characterized by the presence of a J-domain, which is strictly essential for its co-chaperone functions [79,80].

Hsp40/DnaJ interacts with Hsp70/DnaK to enhance its ATPase activity, and thus controls its binding affinity to a substrate protein [79,80]. Like Hsp70/DnaK, Hsp40/DnaJ can bind a nonnative protein and protect it from aggregation. It is thought to recruit a nonnative protein and transfer it to the ATP-bound state of Hsp70/DnaK.

At least one more key-player is involved in the Hsp70/DnaK chaperone system. It accelerates nucleotide (ADP/ATP) exchange in Hsp70/DnaK. In prokaryotes, GrpE performs this [79,80]. It has a molecular mass of 22 kDa and forms a stable dimer in solution. GrpE accelerates/stabilizes the open conformation of the nucleotide binding pocket of the N-terminal ATPase domain of DnaK, which facilitates the release of ADP and binding of ATP [79]. In eukaryotes, BAG proteins and others have been found to act as the nucleotide exchange factors for Hsp70 proteins.

The chaperone cycle of the DnaK chaperone system is summarized as follows [80]. DnaJ binds a substrate, and then interacts with DnaK. The interaction induces a conformational change of DnaK, resulting in stimulation of ATP hydrolysis, and the closing of the substrate-binding cavity. Thus, the ADP-bound form of DnaK exhibits a high affinity for its substrate. GrpE helps release of ADP and binding of ATP from DnaK through its interaction with the DnaK ATPase domain. Upon ATP binding to the N-terminal ATPase domain of DnaK, the substrate-binding cavity becomes opened. The ATP-bound DnaK exhibits a low affinity for its substrate. Thus, the bound substrate is released from DnaK. The chaperone cycle is thought to facilitate protein folding.

#### 20.5.5 Hsp90/HtpG

Hsp90 or HtpG (a prokaryotic homologue), is present in eukaryotic and eubacterial cells. Archaea generally lack genes for Hsp90/HtpG [72]. Members of the Hsp90/HtpG family are present in chloroplast, mitochondrion, endoplasmic reticulum, and are predominantly localized in the cytoplasm [91]. The Hsp90/HtpG family contains both heat-inducible and constitutively expressed members [23,92].

Hsp90 forms a constitutive dimer at physiological temperatures. Each monomer consists of three domains: N-terminal domain, middle-domain, and C-terminal domain. Hsp90 is a very weak ATPase, and its N-terminal domain possesses an ATP binding site [93]. The C-terminal domain is essential for Hsp90 dimerization [94,95]. Structural, biochemical, and mutational analysis of Hsp90 showed that conformational/domain rearrangements of Hsp90 are coupled to the ATPase reaction, which is thought to drive structural changes of a substrate protein and its release [96,97]. Nothing is known about the nature of the changes engendered in Hsp90 clients by association with Hsp90 and passage through the ATPase-coupled chaperone cycle [96].

In eukaryotes, Hsp90 collaborates with co-chaperones/cofactors such as Cdc37, Aha1, and p23/Sba1 to mediate the conformational regulation of a wide variety of substrate proteins including transcription factors and protein kinases under physiological conditions [96,97]. Like the other major classes of Hsps, Hsp90 can recognize and bind nonnative proteins, thereby preventing their nonspecific aggregation [98]. This general protective chaperone function may be especially important under stress conditions. In plant cells, Hsp90-based chaperone hetero-complexes have been identified. Hsp70, a p60/Sti1/Hop ortholog, and high-molecular-weight immunophilins were detected in these heterocomplexes [91,99–101].

As far as we know, there is no direct evidence that Hsp90 is involved in thermotolerance in higher plants. This may be due to the fact that Hsp90 is involved in numerous and diverse cellular functions as described below, and is essential in plants as it is essential for the viability in yeast [26]. The following results with the TU8 mutant suggest that Hsp90 plays a role in thermotolerance in plants [102]. The TU8 mutant of *Arabidopsis* that is deficient in glucosinolate metabolism and pathogen-induced auxin accumulation was found to be less thermotolerant than the wild-type plant. Among different Hsps only the expression level of cytoplasmic Hsp90 declined in the mutant at elevated temperatures. Transient expression of Hsp90 in mutant protoplasts increased their survival rate at higher temperatures to near equivalent that of wild-type protoplasts suggesting that the reduced level of Hsp90 in the mutant may be the primary cause for the reduction in thermotolerance. Although not a higher plant, *htpG* knockout mutants of the cyanobacterium *Synechococcus elongatus* PCC 7942 exhibited great loss of thermotolerance as compared with the wild type strain, indicating that Hsp90/HtpG plays a role in the thermal stress management [103].

Hsp90 has been assigned numerous and diverse functions including protein folding, signal transduction, protein transport across the endoplasmic reticulum and organelle membranes, and protein degradation in the eukaryotic cells [96,97]. One notable function of plant Hsp90 is that it is crucial for defense against pathogens. Hsp90 is involved in plant immunity signaling pathways [104]. The SGT1-Hsp90 pair, a chaperone complex is required for maintenance of immune sensors. Lastly, we should add that Hsp90 buffers genetic variation by keeping mutant proteins in wild-type conformations [105,106]. When this buffering is compromised, for example, by heat stress that diverts Hsp90s from its normal, specific target proteins to denatured proteins, the variations are exposed, resulting in production of an array of morphological phenotypes. The great effect of Hsp90's effects on the buffering and release of genetic variation suggests it may have an impact on evolutionary processes.

### 20.5.6 Hsp100/ClpB

Hsp100/ClpB is present in eukaryotic and eubacterial cells. Archaea generally lack genes encoding members of this family [72]. Hsp100/ClpB has been identified in chloroplast, mitochondrion, and cytosol of plants (Hsp101) [107–109]. Intriguingly it is not found in the cytosol of animal cells [110], while a homologue (Hsp104) is present in the yeast cytosol.

Hsp100/ClpB belongs to the class 1 family of Clp/Hsp100 AAA<sup>+</sup> (ATPases associated with various cellular activities) proteins [111,112]. Hsp100/ClpB forms large hexameric ring structures and contains two AAA<sup>+</sup> modules. Unlike other Hsp100/Clp proteins, Hsp101, Hsp104, and ClpB are not involved in protein degradation. Rather, the members of the Hsp100/ClpB family function as a molecular



chaperone. Hsp100/ClpB neither has foldase activity nor holdase activity. It neither promotes protein folding nor suppresses protein aggregation. It is an ATP dependent unfoldase/disaggregase that mediates the unfolding/dissolving of protein aggregates. The Hsp70/DnaK chaperone system can collaborate with Hsp100/ClpB to refold these nonnative proteins to their native structure.

What is the mechanism for protein disaggregation? A probable mechanism may be as follows [83]. Hsp100/ClpB binds a substrate protein(s) that is subsequently translocated (mechanically pulled) through the central, narrow channel of the Hsp100/ClpB rings in response to ATP hydrolysis. The protein is unfolded during the translocation/the pulling process. This pulling action is associated with unfolding because the substrate protein is forced to enter a narrow channel that cannot otherwise be negotiated. The energy of ATP hydrolysis can be translated by the machine into the exertion of mechanical force needed for unfolding [113]. Upon release from Hsp100/ClpB, the substrate protein is taken care of by the Hsp70/DnaK system. In ClpB, its protein disaggregation activity is potentially associated with the unfolding that occurs during translocation down its central channel was supported by an experiment in which the distal surface of ClpB was engineered so that it interacted with the ClpP protease. This version of ClpB promoted the degradation of disaggregated substrates [114].

Hsp101 is required for acclimation to high temperatures. Successful complementation of yeast *hsp104* mutants with higher plant *hsp101* provided the first clue for their important role in imparting thermoprotection [115–118]. While *hsp101* mutants such as *hot-1* in Arabidopsis were found to be defective in the acquisition of thermotolerance against high temperature [119], the constitutive expression of *hsp101* provided significant growth advantage to the transgenic seedlings at high temperatures [120]. When the expression of *hsp101* was reduced by antisense or co-suppression, the modified plants had impaired acquired thermotolerance [120]. ClpB is also essential for thermotolerance in the cyanobacterium *Synechococcus elongatus* PCC 7942 [121]. These studies with model plants or organisms may prompt someone to construct an improved thermotolerant crop plant by overexpression of Hsp100. High temperature is detrimental to both the vegetative and reproductive stages of rice [122]. Thus development of improved elite rice varieties with enhanced tolerance to high temperature can help in this regard. *A. thaliana* *Athsp101* cDNA was employed for overexpression in an elite *indica* rice cultivar to obtain transgenic plants. 40–45 days old transgenic plants were exposed to 45°C for 3 h and subsequently placed at 28°C for recovery. After 5 days, control plants totally collapsed whereas transgenic plants were green and healthy. Strong accumulation of AtHsp101 was observed in the transgenic lines at normal temperature as well as in response to high temperature stress. On the other hand, native OsHsp100 was significantly induced to almost similar extent in untransformed and transgenic plants at 47°C. Thus, it was speculated that heat-induced OsHsp100 alone was inadequate to impart higher level of protection against heat stress and the constitutive overexpression of AtHsp101 provided the improved thermotolerance capacity to the transgenic plants [123].

In addition to its essential role in acquired thermal tolerance, Hsp101 provides a substantial fitness benefit under normal growth conditions [124]. The T-DNA insertion mutants of chloroplast and mitochondrial homologues showed no evidence for heat stress phenotypes of seedling similar to those observed in *hsp101* mutants [107]. However, the chloroplast homologue was shown to be essential for chloroplast development.

## 20.6 A NOVEL HSP (Hsa32) INVOLVED IN MAINTENANCE OF ACQUIRED THERMOTOLERANCE

A heat-stress-associated 32-kD protein (Hsa32), which is highly conserved in land plants but absent in most other organisms was reported [125]. The gene responds to heat shock at the transcriptional level in moss, Arabidopsis, and rice. Disruption of Arabidopsis Hsa32 by T-DNA insertion resulted in a great loss of thermotolerance that was acquired at 37°C when recovery period at 22°C after

the pre-heat-treatment was longer than 48 h, and severe heat shock was challenged at 44°C. This indicated that Hsa32 is essential for tolerance against a severe heat challenge after a long recovery following acclimation treatment, which is apparently due to a fast decay of acquired thermotolerance in the absence of Hsa32.

## 20.7 ENGINEERING PLANTS TOLERANT TO STRESSES OTHER THAN HEAT

The roles of the Hsps in the thermotolerance were already discussed throughout the above sections. Hence, in this section, we will briefly introduce some publications that are related to the production of plants that are tolerant to stresses other than heat by introducing/inducing an Hsp gene(s).

Temperature stress is not the only stress that leads to the elevated expression of Hsps. The cold induction of some Hsps (for example, see Ref. [126]) may be related to plant defense mechanisms against cold stress. Thus, it is reasonable to expect that the over-expression of Hsps can improve cold stress tolerance in plants. Low-temperature storage is one of the most important methods of reducing postharvest decay and maintaining the organoleptic and nutritional quality of fruits and vegetables. Exposing sensitive fruits to low temperatures induces chilling injury leading to significant changes in overall quality [127]. There is a report showing a simple way to obtain a cold-tolerant tomato fruit by preheat-treatment to induce Hsps. The Fortaleza tomato variety showed high sensitivity to cold storage (87% damage in untreated fruit after 21 days at 2°C). However, when they were heat treated at 38°C in a chamber for 24 and 48 h, the development of chilling-associated symptoms in a significant percentage of fruits (47% and 20% of damaged fruits, respectively) were prevented [128]. They detected increasing accumulation of a 17.6 kDa class I small Hsp in pericarp proteins of the tomato fruit by the heat treatment.

Expression of some Hsps in different organisms has been shown to be affected by a number of chemicals: arsenite [6,14,46], heavy metals such as cadmium, cobalt, copper, nickel, and silver [6,14]. Cytosolic/nuclear Hsc70-1 overexpression in *Arabidopsis* specifically conferred gamma-ray hypersensitivity and tolerance to salt, cadmium, and arsenite [129]. Plants overexpressing Hsc70-1 accumulated less cadmium, thus providing a possible molecular explanation for their tolerance phenotype. DnaK1 from the halotolerant cyanobacterium *Aphanethece halophytice* was overexpressed in the cytosol of transgenic tobacco plants, and was found to improve their salt tolerance [130].

Interestingly, while evaluating researches aimed at developing transgenic crops and plants with enhanced tolerance to naturally occurring environmental conditions it was revealed that the response of plants to a combination of two different abiotic stresses is unique and cannot be directly extrapolated from the response of plants to each of the different stresses applied individually [12]. Plant acclimation to a particular abiotic stress may require a specific response that is tailored to the precise environmental conditions the plant encounters. To illustrate this point, transcriptome profiling studies of plants subjected to different abiotic stress conditions prompted a somewhat unique response and little overlap in transcript expression could be found between the responses of plants to abiotic stress conditions such as heat, drought, cold, salt, high light, or mechanical stress (reviewed in Ref. [12]). Drought and heat stress can be an excellent example of this type, which may occur in the field simultaneously. It was observed that a combination of drought and heat stress had a significantly greater detrimental effect on the growth and productivity of maize, barley, and sorghum as compared with each of the different stresses applied individually [12,131–134]. Transcriptome profiling studies of plants subjected to a combination of drought and heat stress suggest that the stress combination requires a unique acclimation response involving >770 transcripts that are not altered by drought or heat stress [135]. Among them, 11 transcripts encoding Hsps were specifically elevated during a combination of drought and heat stress. Therefore, to develop transgenic crops with enhanced tolerance to field conditions, (over) expression of several Hsps that is tailored to the environmental conditions in transgenic plants may be able to improve the way.

## 20.8 CONCLUSIONS

Hsps are a diverse group of proteins that share the property for the binding of substrate proteins that are in nonnative structural states. They play an essential role in the cellular protein homeostasis. They are involved in a broad array of cellular processes required for both normal cellular functions and survival under stress conditions.

A volume of overwhelming evidence supports the assumption that the Hsps are some of the most important entities to provide heat tolerance to plants. The representative Hsps function as molecular chaperones. They must become even more indispensable at higher temperatures where the probabilities of denaturation, incorrect folding, and aggregation of cellular proteins are much higher.

Genetic manipulation to introduce an Hsp(s) alone may not be sufficient in an attempt to provide a crop plant with traits such as heat tolerance, resistance to dehydration, and tolerance to salt and other stresses. Nevertheless, further understanding of Hsps and molecular chaperones is essential for the future development of such crop plant strains.

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# 21 Effect of Low Temperatures on the Structure of Plant Cells: Structural, Biochemical, and Molecular Aspects

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## 21.1 INTRODUCTION

Among the external factors that greatly affect cell development is temperature. A favorable temperature has a positive effect on structural and physiological processes of plant cells. When the temperature is increased or decreased, a harmful effect on the plant cells can be observed. Plant sensitivity to temperature depends on the plant's origin and phylogeny. The effect of temperature on cell ontogeny has been extensively studied and there are reviews elsewhere concerning this topic [1,2].

In this chapter we have tried to submit the results regarding the effect of low temperatures on the structure of the plant cell. Mutual comparison of existing results and their generalization is not easy, because variable plant species in different ontogenetic phases have been used in the observation.

The temperatures on the Earth's surface are very different, changing during the seasons as well as during the day and nights. Despite these differences, the plants grow almost everywhere. However, to be able to survive the unfavorable temperatures, plants have to adapt to this temperature oscillation. Plants, on the basis of their sensitivity to temperature, have been divided into three groups [1]:

1. *Chilling-sensitive plants*: These plants are seriously injured by the temperatures above zero (usually below 15°C).
2. *Chilling-resistant plants*: These plants are able to tolerate low temperatures but are seriously injured when ice start to form in their tissues.
3. *Frost resistant plants*: These plants are able to tolerate exposure to very low temperatures (of -50°C to -100°C even when immersed in liquid nitrogen).

Most perennial plants growing in the temperate regions undergo a "hardening" process in the autumn of each year to prepare for overwintering. In most agricultural areas, unseasonal frost can occur throughout much of the growing season. During periods of active growth, most crop species do not tolerate freezing. Depending on the minimum temperature and the duration of the frost, plants may be partially damaged or killed, resulting in lower yield and quality of harvest or even complete crop failure. Most winter crops, however, have the ability to develop freezing tolerance when exposed to hardening conditions.

Each plant is characterized by certain genetically fixed level of resistance to low temperatures, which reduces its metabolic activity at such low temperatures. This level of resistance (or survival capacity) can vary among individual plant species. Low temperatures act as a stress factor that has a strong impact on growth, reproduction, and distribution of plants. The ability of plants to survive and grow depends on different ecological and physiological mechanisms [1–4].

## 21.2 CHILLING

Chilling injury can be observed on many plants of tropical and subtropical origin when they are exposed to low, but nonfreezing temperatures, in their chilling range, which is usually from 25°C to 10°C [5]. For plants of temperate origin, chilling temperatures usually range from 15°C to 0°C. The chilling effect is manifested by physiological and cytological changes. Depending on the time and temperatures, the cytological changes can be either reversible or irreversible. However, chilling-sensitive plants are also able to adapt to the chilling if they are hardened a certain amount time at temperatures slightly above their critical temperatures.

Many light- and electron microscopic studies have shown different structural changes of the cells in chilling-sensitive plants after their exposure to a long period of chilling stress [6–8].

### 21.2.1 CELL MEMBRANES

The cellular membranes are those cell compartments, where the primary events of chilling stress occur [9]. An increase in the permeability of the plasmalemma and leakage of organic and inorganic

substances is considered to be the first symptom of cell injury [10]. Light- and electron microscopical observations of tomato cotyledons growing at 5°C for 3 days have revealed the loss of cell turgor, vacuolization of cytoplasm, swelling, and disintegration of cell organelles [11]. More detailed ultrastructural time-course studies have shown injury of plasmalemma after 20–24 h. Disintegration of the plasmalemma can be observed after prolonged cold treatment or at lower temperature [12,13].

During plasmolysis of hardened and nonhardened cells of rape and alfalfa plants, the plasmalemma is pressed against the tonoplast and deleted into the vacuole as sac-like intrusions [14]. A similar sac-like invaginations of the tonoplast into the vacuole during hardening at 5°C can be seen in potato leaves [15].

Chilling of the roots of the tropical plant *Episcia reptans* results in tonoplast discontinuity within 1 h at 5°C and 3 h at 10°C [16]. Two types of crystalline deposits (cytoplasmic and tonoplast-associated) are seen in root cells after chilling stress. Since similar deposits have also been observed in epidermal, mesophyll, and vascular cells of *E. reptans* leaves [17] and on the tonoplast of potato cotyledons [13], and these deposits closely follow tonoplast disruption, it can be supposed that these deposits probably serve as an indication of cell injury in the plants with increased time of exposure. Although the injury of a majority of the membranes after a short period of chilling is usually reversible, injury of the tonoplast is irreversible [18] and may govern the ability of plants to survive rewarming [19].

Frequently, as a result of chilling stress or hardening at low, above-zero temperatures, lipid bodies accumulate in the cytoplasm or in close association with plasmalemma [20,21].

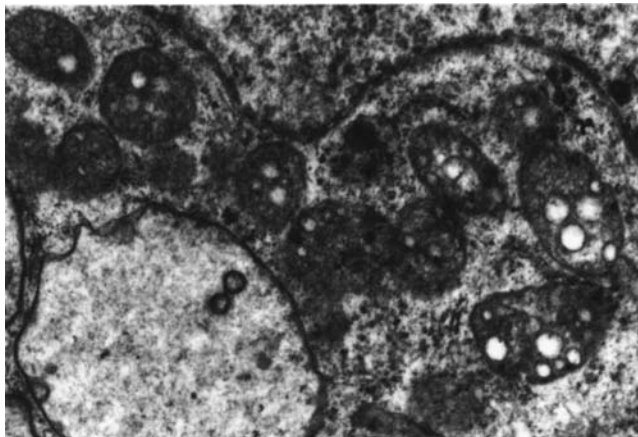
### 21.2.2 PLASTIDS AND MITOCHONDRIA

Swelling of plastid membranes and mitochondria is a very common symptom of chilling temperatures. The harmful effect of these temperatures is mostly time dependent. Chloroplasts from the leaves of *E. reptans* chilled for 6 h at 5°C have an irregular and less organized membrane system and fewer plastoglobules. Increase in the exposure time results in both swelling of the chloroplast thylakoids and in a decrease in the size and number of starch grains [17]. After 4 h of exposure at 5°C, injured chloroplasts disintegrated thylakoids can be seen [11]. Full grana disintegration and an increase in the number and size of the plastoglobuli can be observed in hardened cucumber leaves after 11 days of chilling. Hardening of potato leaves for 10 days at 5°C causes dilation of the thylakoids and the disappearance of starch grains [15]. The chilling stress induces the reduction of starch grains and thylakoids in winter wheat and in maize [22]. When *Ephedra* cells are cultivated for 15 days at 2°C, the plastids together with mitochondria are organized into groups. Plastid grana are innumerable and plastids very often contain membrane-free stroma [23].

Contrary to this result, the long-term hardening of young seedlings of Norway spruce at 3°C increases the content of the starch grains in plastids and the thylakoids are not distinctly dilated. Such plastids possess numerous plastoglobules [20].

After the 1-day exposure of *Ephedra* cells to 2°C, the mitochondria have less dilated cristae and their matrix is transparent [23]. Swollen mitochondria with reduced cristae have been observed in chilled onion cells [24], in maize root cortex ([Figure 21.1](#)) [8], and in both root and leaves cells of *E. reptans* [16,17]. Owing to the mitochondrial swelling in chilled tissues, their volume is doubled in comparison with the mitochondria from the plants grown at a favorable temperature [17]. The first symptoms of visible injury of mitochondria have been recognized after exposure of tomato cotyledons at a temperature of 5°C for 4 h [13]. These mitochondria possess a reduced number of cristae and discontinuities in their envelope. Structural alterations of mitochondria have been seen in the microsporocytes and tapetum of *Rhoeo discolor* exposed to a temperature of 4°C–5°C for 4 days [25]. In contrast, no visible changes in the mitochondria have been detected in xylem ray cells of poplar trees at a temperature of 0°C for 14 days [26].

Recently, an electron microscopy study has shown that the mitochondria in the axis of soybean seeds imbibed at 10°C and 4°C were slightly diminished and lacked internal structure. In contrast,



**FIGURE 21.1** Mitochondria with dilated cristae from maize root cortex cells at 5°C (×24,000). (From Čiamporová, M. and Mistrík, I., *Plant Cell under Unfavourable Conditions* (in Slovak), Veda, Bratislava, 1991. With permission.)

the mitochondria in the axis imbibed at 22°C contained numerous well-developed cristae with easily distinguishable outer and inner boundary membranes [27].

### 21.2.3 ENDOPLASMIC RETICULUM AND DICTYOSOMES

The endoplasmic reticulum (ER) of plant cells seems to be very sensitive to cold. A strict correlation has been found between the temperature (between 30°C and 5°C) and the volume of the ER labyrinths. After exposure of plants to cold, an extensive dilation and vesiculation of smooth ER cisternae can be observed quite clearly, and the profiles of the rough ER almost completely disappear with the drop in temperature [28]. These dilated vesicular ER cisternae probably serve as accumulation sites of cryoprotective substances [26].

Prolonged exposure of *Cornus stolonifera* callus cells to 0°C for 12 h results in partial dilatation followed by microvesiculation of the rough ER and releasing of the ribosomes from the membranes. Vacuolization of smooth ER is visible after 24 h of chilling [18]. Dilation of rough ER without ribosomes has been observed in cooled microsporocytes [25]. The vesicles originating from the dilated rough ER without ribosomes have autolytic functions in chilled cells [11,12]. It might be suggested that the transformation of the rough ER into vacuolated smooth ER represents an early stage of chilling [18].

Full reversibility of the ultrastructural changes has been shown suggesting that the ER system is very dynamic; it is probably the most dynamic structure in plant cells [28]. Many studies have shown that the formation of parallel and concentric layering of ER cisternae can be induced by different types of stress and therefore it might have been suggested that these configurations are manifestation of an adaptive mechanism protecting the plant cells and of repairing processes within stress-damaged cells [29,30].

Dictyosomes are cell organelles that are metabolically very active, for example, in protein sorting, and membrane formation. They respond to chilling stress by swelling. The swollen dictyosome cisternae occur in tomato cotyledons after 4 h of chilling at 5°C [13] or after 24 h at 0°C in *Cornus stolonifera* cells [18]. During cold hardening of *Arabidopsis thaliana* cells contain more microvesicles that are either associated with dictyosome cisternae or located in their vicinity; the dictyosomes probably take part in structural and conformational modification of plasmalemma [31]. Longer exposure to chilling temperature causes disintegration of the dictyosomes [32,18].

#### 21.2.4 NUCLEUS

Numerous studies of the effect of chilling temperatures from 0°C to 4°C on the functional and structural behavior of nuclei in pollen mother and tapetal cells of *R. discolor* have been done. A short treatment of nuclei with cold does not cause any important changes in morphology of the nuclei and in DNA synthesis [33]. However, a longer cold treatment considerably reduces both DNA and RNA synthesis [34].

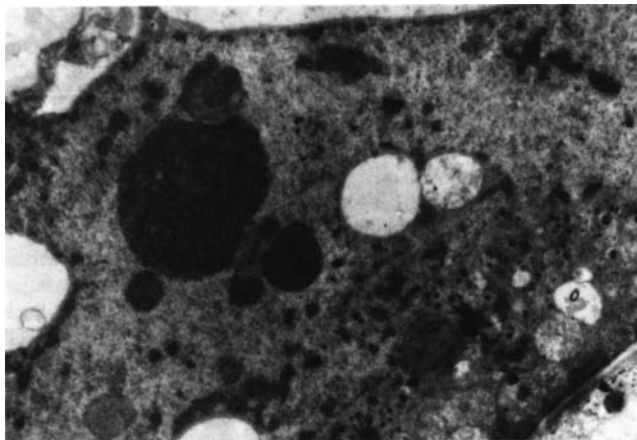
Modification of the nuclear structure of plant cells has been observed by both light microscopy and electron microscopy. Lobed nuclei in the *Ephedra* cells have been observed after long-time (15 days) exposure to 2°C [23]. The nuclei of other plants respond to longer exposure to low temperatures by swelling and modification of the nuclear envelope [3,25] and chromatin coagulation [37]. Following cold stress at 5°C for 3 days, the nuclei in the root cells of maize contain rather dispersed chromatin, nuclear bodies often occur in the nucleoplasm, and the nucleolar organizer regions are pronounced (Figure 21.2) [35,36].

Fully nuclear disruption is observed in *C. stolonifera* cells and in very sensitive *E. reptans* cell after 2 days at 5°C [16,18]. In tomato cotyledons, irreversible injury of nuclei is seen after 20–24 h of chilling [13]. After exposure of wheat cells to the chilling temperatures, the fibrillar zone of the nucleolus is more abundant and granular zone becomes diffused [37,38]. A high amount of fibrillar components can result in the formation of nucleolus-like bodies in the cytoplasm [39] or in the nucleoplasm [8,22,36].

Besides the nucleus structure, a decrease in temperature from the optimum value to the minimum value (about 1°C) is accompanied by a progressive slowdown of the mitotic cycle as well as of the duration of mitosis. At the temperature of 3°C, for instance, the mitotic cycle in *Vicia faba* root cells may be 22 times longer than at 25°C [40].

#### 21.2.5 CYTOSKELETON

According to the some investigators [6], the primary response to the chilling stress may be a breakdown of cytoskeleton, and alterations in membranes, that is, a physical phase transition of membranes from liquid-crystalline to solid gel state [9]. It has been suggested that chilling stress has a direct effect on the microtubules [41]. This major component of the cytoskeleton, have been found to depolymerase during cold treatment [42,43]. Disassembling of microtubules in response to low temperatures depends on the type of organisms and reflecting critical temperature. In chilling sensitive



**FIGURE 21.2** Nucleus with a nucleolus, pronounced nucleolus organizer region, and nuclear bodies in the nucleoplasm from maize root cortex cells at 5°C ( $\times 18,000$ ). (From Čiamporova, M. and Mistrík, I., *Environ. Exp. Bot.* 33, 11, 1993. With permission.)

plants this disassembly start as early as at temperatures above 4°C, whereas in moderately resistant plants they can withstand 0°C; in cold-resistant species disassembly did not occur even at temperatures below -5°C [41]. Depolymerization of cortical microtubules at chilling temperatures (0°C–4°C) has been repeatedly observed in several chilling-sensitive species of higher plants [42] and in various cell types, including the root cells of maize [45], the guard cells of onion [46], the suspension culture cells of maize [47], and protoplasts isolated from tobacco [48].

In experiments with cucumber cotyledons, it has been found that treatment with antimicrotubular drugs make the chilling worse, whereas treatment with abscisic acid (ABA) protects cotyledons from drug effect and chilling injury [49].

There is a connection between chilling of cytoskeleton and the inhibition of cytoplasmic streaming. The chilling temperatures can influence the equilibrium of  $\text{Ca}^{2+}$  and adenosine triphosphate (ATP), which is connected with F-actin activity [6,7,50]. Actin filaments have also been found to be involved in cold-induced conformational changes and the reorganization of the ER [51]. These results indicate that low temperatures (0°C–4°C) most likely influence either the interaction of the force-generating system, probably myosin, with actin filaments or the force-generating mechanism of the actomyosin-driven intracellular movement, but they do not affect actin-filament integrity [51].

The effect of low, nonfreezing temperatures on the plants also is visible at anatomical and morphological levels. These aspects are connected mainly with adaptation reactions of less chilling-sensitive and cold-resistant plants (like winter cereals) to growth at low temperatures. Such anatomical and morphological changes like altered stomatal frequency [52], decreased epidermal cell size [53], increased mesophyll cell size, and suberization [52,54] are associated with acclimation of plant to nonfreezing temperatures.

### 21.3 FREEZING

Generally, freezing in plants consists of the conversion of liquids in cells to a solid state, which is accompanied by loss of heat. Two types of freezing are recognized in plant cells and tissues: (a) vitrification—solidification of cellular content into noncrystalline (amorphous) state and (b) crystallization—arrangement of liquid molecules into orderly structures [55]. Vitrification of liquids in cells is a result of rapid freezing (at more than 3°C/min) of plant tissue to very low temperature. It is enhanced by hardening of plants at low temperatures. Although vitrification does not occur in nature, it is of great interest to researchers because it enables plants to survive temperatures close to absolute zero [56].

On the other hand, crystallization (or ice formation) is a very common phenomenon in nature. The crystallization of ice may occur either within or outside the cells, but the process depends on the speed of cooling. The formation of ice inside the cells may occur by both internal nucleation or by penetration of external ice crystals into the cells [57]. In both cases, this type of freezing, also called intracellular, is lethal because of the immediate disruption of the cells. The only exception of this rule may be in case of the cells that exhibit deep supercooling [58]. Plant cells can also survive intracellular ice formation when the ice crystals that form by freezing are very fine, cooling is extremely rapid, and these crystals melt before they reach a harmful size [59].

There are three types of intracellular ice formation in epidermal cells of onion plants at high-speed of cooling [60]: (a) Ice formation spreads from cell to cell through the plasmodesmata. Freezing from cell to cell is also observable on the *Tradescantia* staminal hair cells [61] and in mesophyll cells of Norway spruce during the winter frosts [62]. (b) Less frequently, ice can be formed in the cell walls adjacent to the intercellular spaces. Ice arises first in the plasmolyte between the cell wall and cytoplasm and then rapidly in the cytoplasm. (c) Intracellular ice originates spontaneously from centers of nucleation within the cytoplasm and later in surrounding plasmolyte.

If the speed of cooling is slow enough (in nature, the cooling rate seldom exceeds 1°C/h), the liquids in the cells freeze extracellularly, causing cell dehydration of cytoplasmic solutes and a reduction in cell volume and surface area, all factors which can potentially damage the

cells irreversibly [63]. Ice formation for most plant tissues begins on the surface of cell walls, in water transport elements, or on external surfaces. Although the cooling is slow and plasmalemma remains intact, ice formation will be confined outside of cells [64].

There are two major strategies allowing plants to survive freezing stress: freezing tolerance and freezing avoidance [2]. Tissues displaying freezing tolerance respond to freezing stress by the loss of cellular water to extracellular ice, resulting in collapse of the cell. As a consequence, an increased concentration of the cell sap and a lowered freezing point will occur. Cell wall structure, rigidity, and porosity play important roles in the capacity for extracellular and extra-organ freezing [65]. The control of ice formation and movement of water from the protoplast to extracellular spaces appears to be particularly important in nonconiferous plants [65]. For work which has been done on conifers, see [66].

In plants displaying second strategy—freezing avoidance, tissues exhibit deep supercooling, in which cellular water is isolated from the dehydrative and nucleating effects of extracellular ice [67].

The formation of ice in tissues and the appearance of frozen plant cells is well documented, mainly in studies employing light microscopy [56,61]. Descriptions of frozen cells at the electron microscopic level also have been done [68].

### 21.3.1 CELL MEMBRANES

As already mentioned [68], functionally intact cell membranes are an effective barrier to the propagation of ice; however, this barrier may vary depending on the temperature or cold hardening [69]. Although the mechanisms involved in plant cold acclimation and frost injury are extraordinarily complicated, the freezing and thawing of cellular water have been found to be basic elements of freezing injury in plant tissues [57]. It has been established that the cellular membranes are more susceptible to freezing damage than soluble enzymes. The plasma membrane seems to be the most susceptible and, therefore, it has been identified as the major site of lethal injury [70].

Leakage of ions from thawed tissues is a common phenomenon of freezing injury. The leakage is usually considered the consequence of the loss of membrane semipermeability or membrane rupture by freezing injury. However, observations on onion epidermal cells indicate that freezing injury is firstly due to a specific alteration in the membrane semipermeability to  $K^+$ , and secondary effect is protoplasmic swelling [71].

There are numerous studies dealing with the physiological and biochemical changes occurring in membranes during the freezing and cold hardening processes [4,70,71,74] but observations regarding alterations in the cellular membranes are rather insufficient [59,68,72,73].

Isolated plant protoplasts are an excellent model system to study destabilization of the plasma membrane after freezing stress. The use of protoplasts has shown that destabilization manifests in various ways: by intracellular ice formation, by loss of osmotic responsiveness, or by expansion-induced lysis [69]. If cellular membranes are the site of freezing injury, then cellular alterations during cold acclimation that allow the cells to survive freezing also will appear in membranes [74].

Cold acclimation involves chemical and structural alterations of the plasma membrane to resist freeze dehydration, mechanical stress, molecular packing, and other events caused by extracellular freezing. Cytological changes associated with an abrupt increase in hardness occur at  $0^{\circ}\text{C}$  or  $-3^{\circ}\text{C}$  within 7–10 days. However, these changes may be indirect.

Observations on *Robinia pseudoacacia* have revealed a seasonal transition in the plasmalemma from a physical state of relative smoothness and regularity in summer to a highly folded state in winter. It is considered that a highly folded membrane state would facilitate water flow and alleviate the stresses of contraction and expansion during freeze–thaw cycles [75]. However, the plasma membrane of cortical cells of mulberry twigs in winter is relatively smooth, and highly folded states have not been observed. Only after cold acclimation in October at  $0^{\circ}\text{C}$  for 20 days or  $-3^{\circ}\text{C}$  for 7 days, when hardness increased at temperatures ranging from  $-15^{\circ}\text{C}$  to  $-70^{\circ}\text{C}$ , was the plasma membrane highly folded and microvesicles with a double lipid layer membrane appeared

in the peripheral cytoplasm. These microvesicles originate from the ER. A very similar ultrastructure has been observed in the cold-acclimated cells collected at the end of autumn. In April, at a decreased hardness at  $-15^{\circ}\text{C}$ , the plasma membrane is already smooth and regular. When these dehardened cells are rehardened at  $0^{\circ}\text{C}$  for 10–15 days, hardness increased, the plasma membrane becomes folded, and microvesicles reappear near the periphery of the cytoplasm. From these results, it appears that a highly folded state of the plasma membrane and the formation of numerous microvesicles represent a transition associated with higher freezing tolerance rather than representing a special membrane structure characteristic for extremely hardy cells in the winter state [76].

Formation of osmiophilic regions associated with the plasmalemma has been also observed. Substantial regions of the plasmalemma bilayer are transformed into either amorphous, osmiophilic or densely packed regions or in multilayered structures with high surface curvatures [77]. Deep invaginations of the plasmalemma and formation of electron-dense deposits outside the plasmalemma in xylem parenchyma cells of peach and oak trees in a frozen state at  $-10^{\circ}\text{C}$  occur [67]. We have observed similar changes of plasmalemma in the mesophyll cells of silver fir [106] and numerous electron-dense lipid bodies associated with the plasmalemma in mesophyll cells of Norway spruce during winter, when the frost resistance of these species is very high [62]. Augmentation of lipidic globules and their localization in the cytoplasm along the plasmalemma apparently results in the changes in lipidic part of membranes during the freezing treatment [78].

It has been found [79] that osmotic shrinkage of protoplasts isolated from *Secale cereale* results in an irreversible decrease of the surface area of the plasmalemma concurrent with the formation of endocytotic vesicles. This may lend support to the idea that the reduction of the plasmalemma surface area and the reduction of the volume of the protoplast through dehydration occur as initial responses to slow freezing [14,79].

Increasing of the intramembranous particles and plasmalemma invaginations has occurred in more frost-resistant *Chloromonas* cells, whereas in the frost-sensitive *Chlamydomonas* cells, they are absent [80]. A higher frequency of osmiophilic globules in acclimated ( $-25^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$ ) isolated protoplasts of *S. cereale* have been found than in nonacclimated ( $-3^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$ ) protoplasts. Osmiophilic regions observed under transmission electron microscopy corresponded to the extrusions of the surface of acclimated protoplasts observed under scanning electron microscopy (SEM) [69].

SEM observations on apple parenchyma cells have revealed similar copula-shaped protrusions on the surface membranes. The protrusions are associated with the fibrillar formations of exoplasm. It is clear that mechanical breaks of membrane may arise on the plasmalemma near protrusions under stress conditions of freezing. It can be supposed that plasmalemma instability zones are formed under freezing stress connected with protoplast compression under dehydration, whereas protrusions themselves consist of structural lipids of higher unsaturation. Intracellular processes leading to the membrane stabilization are evidently related to condensation of polyphenols, which make cell resistance under stress conditions at super-low temperatures essentially higher [81].

If plants are nonacclimated or the freezing stress is very severe, disruption of the plasmalemma and cell organelles and the collapse of the cell wall with the protoplast can occur [21,82]. In case of the moss, *Physcomitrella patens*, when frozen to  $-4^{\circ}\text{C}$ , freezing injury-associated ultrastructural changes such as formation of nontreated aperticulate domains and fracture-jump lesions were frequently observed in the plasma membrane of protonema cells but not in that of ABA-treated cells [82].

### 21.3.2 ENDOPLASMIC RETICULUM

If the plasmalemma is considerably damaged, its protective function against quick dehydration of cells or penetration of ice into cells can be replaced by parallel layering of the ER [84]. The ER is structurally and functionally highly dynamic part of the endomembrane system of plant cells. The response of the ER is immediate at the low temperatures, which is accompanied by shift in its structure and space organization in the cells.



One of the specific features of wintering plants is the absence of rough ER in the cells. This type of ER, observed in the cortical cells of apple during the growing season, at freezing temperatures in winter becomes sparse and replaced by vesicular ER [85]. The cells enriched with numerous tubular and vesicular smooth ER cisternae have also been observed in ray parenchyma cells of poplar. These smooth ER cisternae are the most characteristic components of cells in the winter stage, and they are suspected to be the site of sugars accumulation [86]. In the cells that survive freezing temperatures by a deep supercooling mechanism, the presence of tubular ER is a feature of dehydration tolerance [87].

The ultrastructural study of such extremely cold hardy cells such as cortical parenchyma cells of mulberry collected in winter has shown that initiation of freezing at 5°C results in the formation of multiplex lamellae that completely cover the area in the vicinity of the plasmalemma. The multiplex lamellae are produced by fusion of preexisting vesicular ER via a reticular network. The completed multiplex lamellae are composed of a parallel array of sheet-like ER cisternae. The formation of multiplex lamellae on the initiation of freezing is largely dependent on seasonality in close association with the development of freezing tolerance [84,88].

Examples of stacked ER were also found in dormant buds in potato and in several other species, mainly trees, such as *Betula* [89], *Sorbus*, *Quercus*, *Fraxinus* [89,90], *Rhododendron* [91], and *Salix* [92]. The stacking of ER disappears in spring in connection with breaking of dormancy. In a study of dormant *Tilia* buds, using freeze-fractured material, no concentric layering of ER was observed, but an extensive network of ER close to the plasmalemma was found [93].

The groups of stacked ER cisternae have been observed in cells of wheat seedlings at -10°C, whereas at -30°C, ER has been present in the form of numerous vesicles and sacs [94]. The presence of numerous vesicles and cisternae of smooth ER close to the cell wall is considered to be a characteristic feature of frost-resistant cells [68,75,84]. The occurrence of the concentric type of rough ER in frozen cells is an adaptive mechanism protecting ribosomes against injury by low temperature [32].

### 21.3.3 VACUOLE

The vacuolization of the cytoplasm is a very important phenomenon, and it is often described as a structural reaction of cells to freezing. Reversible splitting of the large central vacuole into many smaller ones has been observed in many plants; namely, woody species. At the beginning of cold acclimation of peach stem tissue, the cells have their typical architecture—a large central vacuole and a thin band of peripheral cytoplasm—but with the continuing cold acclimation, distribution of the cytoplasm gradually becomes more homogenous, that is, the nucleus is located centrally and many small vacuoles appear in the cells [67]. Splitting of the central vacuole has been recorded in the mesophyll cells of *Pinus cembra* and *Picea excelsa* after the first autumn frosts [44,83], in the phloem cells of *Metasequoia glyptostroboides* [32], and in the mesophyll cells of Norway spruce and silver fir during winter period [62].

A dense and extensive cytoplasm containing numerous small vacuoles is characteristic for winter-hardy cells. Autophagic activity of the vacuoles after severe cold injury has been observed in many plant cells, which results in the digestion of cytoplasmic structures and reorganization of distinct cytoplasmic organelles. The release of protein-toxic vacuolar substances resulted in frost injury of spruce needles due to loss of cell compartmentation and concomitant flooding of the cell interior [32]. Functional stability of the tonoplast, therefore, can play an important role in the frost resistance of spruce needles.

Seasonal changes in the vacuole from winter to spring in mulberry cortical cells consist of an engulfment of the tonoplast, fusion and inflation of small vacuoles, and coalescence into larger vacuoles [75]. Similar findings have been seen in the mesophyll cells of both Norway spruce and silver fir [62,106] and in the leaves of evergreen species *Aucuba japonica* and *Prunus laurocerasus* [21]. Decay of the central vacuole to small vacuoles is an adapting mechanism of the plants to low

temperatures in autumn and in winter, although this process of adaptation is reversible. With the increasing temperatures in spring, the central vacuole is differentiated again by the fusion of small vacuoles [95].

On the other hand, although the splitting of the central vacuole is also observed in other woody trees (e.g., *Sambucus* and *Betula*) at temperature  $-30^{\circ}\text{C}$  to  $-50^{\circ}\text{C}$ , this phenomenon can be considered the consequence of a decrease in frost resistance [84]. A decrease of frost resistance in the cells of *Robinia pseudoacacia* also results in degradation of cell membranes, including the tonoplast [96].

Vesiculation of the tonoplast into the vacuole may represent a similar mechanism (like vesiculation of the plasmalemma) for the reduction of the surface area of the tonoplast and the volume of the vacuole. Observations using osmotically manipulated isolated cells of *Brassica napus* and *S. cereale* support this assumption [14].

#### 21.3.4 CHLOROPLASTS

The response of chloroplasts to low-temperature stress depends on the temperature and hardening capacity of particular species. Numerous data from some extremely hardy conifers and from a few moderately frost-resistant herbaceous plants indicate variable changes in the chloroplast membranes in different species [97]. For instance, coniferous species tolerate temperatures at around  $-40^{\circ}\text{C}$  (and lower), whereas moderately frost-resistant plants such as winter annual herbs and grasses are already killed at  $-10^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ .

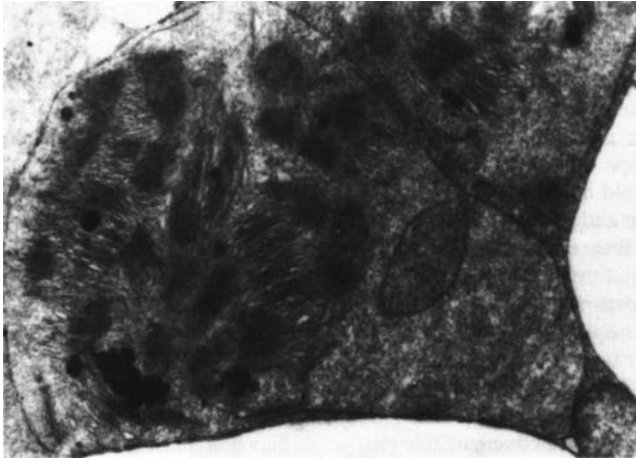
It is often assumed that the chloroplasts are the cell organelles most sensitive to low temperatures [32]. Observations of three grass species have been shown that the transition from  $25^{\circ}\text{C}$  to low-temperature conditions ( $10^{\circ}\text{C}$ ,  $0^{\circ}\text{C}$ ,  $-5^{\circ}\text{C}$ ) causes the swelling of chloroplasts in mesophyll cells at  $0^{\circ}\text{C}$ . Dilatation of thylakoids has occurred at  $-5^{\circ}\text{C}$ . Similar structural changes of chloroplasts and the disappearance of starch grains were observed in the mesophyll cells of *Sorghum* and *Paspalum* [98], and in winter wheat [95]. Chloroplasts of resistant wheat varieties [99] and winter rape [100] change their shape during the hardening, even as retaining their individuality, but during winter they are clumped together.

After rewarming during the spring, the structural recovery of the chloroplasts can be observed [100].

According to the position of chloroplasts in the cells during freezing stress, plants can be divided into two groups: (a) the chloroplasts retain their integrity but migrate from a summer position near the cell wall to a crowded position in the cell center and (b) the chloroplasts agglutinate, lose their integrity, and merge with each other to become a continuous mass from which the chloroplasts separate again when spring approaches [56].

Observations on broadleaf evergreen woody species as *A. japonica* [101], *P. laurocerasus* [102], *Skimmia japonica* [103] and *Mahonia aquifolium* (unpublished observations) have revealed remarkable changes in the chloroplast structure during the year. In summer, chloroplasts are oval shaped, they are placed along the cell walls, and their inner architecture is the same as in other higher plants [104] and contains starch grains. In autumn, the originally lens-shaped chloroplasts of *Aucuba* and *Skimmia* become globular and move gradually from the cell wall to the center of the cell. The chloroplasts of *Prunus* and *Mahonia*, which are more frost resistant than *Aucuba* and *Skimmia*, are still positioned at the cell wall. At this season of year, no starch grains have been observed in the chloroplasts of the plants studied [105].

In winter, the chloroplasts of *Prunus* are still distributed along the cell wall, whereas the chloroplasts of *Skimmia* and *Aucuba* create irregular formations in a different part of the cell. The well-developed membrane system with the signs of slight dilatation can be observed. The membrane system of the chloroplasts is often located in one part of chloroplast, leaving only membrane-free stroma in the other part (Figure 21.3). In the chloroplast stroma, small groups of plastoglobuli are present and no starch grains can be visible. Aggregation of the chloroplasts around the nucleus is a

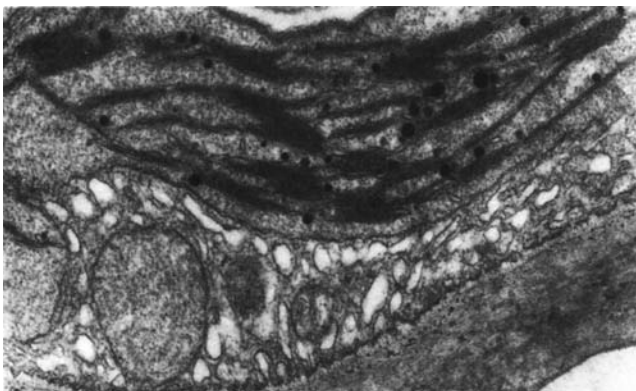


**FIGURE 21.3** Chloroplast of *Aucuba japonica* in winter with membrane-free stroma and slight thylakoid dilation ( $\times 22,000$ ). (From Hudak, J. and Salaj, J. *Photobiochem. Photobiophys.*, 12, 173, 1986, With permission.)

phenomenon connected with the winter metabolism of the cell and is a characteristic feature of the most frost resistant species [83].

A characteristic feature of the chloroplasts of broad leaf species in the spring is the presence of a large number of starch grains resulting in irregular plastid shape. These plastids represent an atypical stage within the plastid ontogenesis chloroamyloplasts [104]. Similarly large content of starch is also observed in the cells of *Abies alba* and *Picea abies* [20,106]. Reports have confirmed that the plasticity of the chloroplast membrane system enable the cells to overcome unfavorable conditions during the winter.

Mistletoe is well-known semiparasitic plant in which its leaves exhibit a high level of frost resistance. We have not found any striking differences in the ultrastructure of *Viscum* chloroplasts in the winter and summer. The thylakoid system of chloroplasts is composed of numerous grana and stroma lamellae. Both the stroma lamellae and marginal thylakoids of grana show signs of slight dilatation. The membrane system remains well differentiated even at a temperature as low as  $-7^{\circ}\text{C}$ . Small plastoglobuli and a low amount of starch grains are regularly distributed in stroma. The chloroplasts are regularly sheathed by membranes of the ER during the summer, but in the winter, these membranes are fragmented into vesicles of variable shape and size (Figure 21.4) [107].



**FIGURE 21.4** Mistletoe chloroplast with an extensive fragmentation of endoplasmic reticulum into vesicles at  $-7^{\circ}\text{C}$  ( $\times 19,000$ ). (From Hudak, J. and Lux, A., *Photosynthetica* 20, 223, 1986. With permission.)

The chloroplasts of conifers [108,109] respond to cold acclimation and freezing by extensive changes in their architecture and localization in the cells. Generally, the chloroplasts of conifers respond to low temperatures mainly by a reduction of starch content, with the increase in the number of osmophilic globules and of membrane-free stroma, and by swelling of the chloroplasts and their aggregation in one part of the cell [20,62,110]. Similar studies have been done on white spruce [111], balsam fir [112], and Scots pine [110].

The significant presence of numerous starch grains, incorporated into thylakoid system, has been recorded in all investigated evergreen species in the spring, when the chloroplasts are again less frost resistant. This, so called “spring starch” serves as a source of energy for the growth processes in this period [101,102,108]. On the other hand, the disappearance of the starch from the chloroplasts during cold acclimation, even in ABA-treated plants [82] is a typical reaction of both broadleaf and coniferous evergreen species [108,111], as well as of the cells cultivated in vitro with decreasing of temperature [20,113]. The hydrolysis of starch is one of the basic physiological mechanisms for the increase in the frost resistance of plants [114].

### 21.3.5 NUCLEUS

Although the nucleus, because of its regulation of cell metabolism is considered to be the cell organelle most resistant to the nonlethal effect of low temperature [115], there are not many studies concerning its structural response to the freezing stress and to the process of developing frost resistance. The nuclei in the cells of black locust bark become denser during cold acclimation in the autumn [116]. The nuclei of the acclimated cells in the shoot apex of the rhododendron are ovoid and contain relatively large nucleoli and little heterochromatin, or they are irregularly shaped with small protruding lobes or nucleoplasmic extensions [91]. In the cortical cells of apple [85] at the stage of cold acclimation, each nucleus contains relatively lower amounts of heterochromatin and is located in the central part of the cell.

The cooling of tobacco cells to  $-10^{\circ}\text{C}$  induces formation of numerous small vacuoles in the nucleus [117]. Similar vacuolization of nuclei also have been observed in the cells of the wheat leaves at  $-4^{\circ}\text{C}$  after 8 days of freezing, whereas in the nuclei exposed to  $-12^{\circ}\text{C}$ , vacuoles already occur after 1 day [118].

The second step of wheat hardening at  $-16^{\circ}\text{C}$  results in folding of the nuclear membrane and condensation of the chromatin [95]. Heterochromatin condensation seems to be a common reaction of the nuclei to a freezing temperature both in perennial grasses [72,118] and in woody plants [62,119]. In the mesophyll cells of spruce, in addition to nuclei with condensed heterochromatin, large nucleoli and changed nuclear membrane also occur during winter at  $-10^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  [62]. During winter, the nuclei move to the central position of the cells and are surrounded by the aggregations of the swollen chloroplasts. The movement of nuclei toward a central position in the cortical cells of apple during cold acclimation also has been observed.

### 21.3.6 MITOCHONDRIA

The mitochondria are the primary site of intermediary metabolism in the cell and are therefore an excellent means to the study of plant response to the changes in the environment [120]. The alterations of the mitochondrial membranes directly influence the process of cell respiration.

Decreasing the temperature in the environment is accompanied by a decrease in the number of mitochondrial cristae and the matrix becomes electron transparent [76,84]. Swollen mitochondria and a reduction in the number of the cristae or their atypical orientation in the cell have been found in the dormant buds of *Salix* in early winter [92] and in the shoot apex cells of *Rhododendron* [91].

The reaction of herbaceous plants to freezing stress is similar to that seen in woody species. The mesophyll cells of winter rape in October at the temperature of  $-6^{\circ}\text{C}$  possess mitochondria with reduced number of cristae and low electron density of the matrix. In December, when the cells are

highly injured (about 80%), the mitochondria are hardly visible because of their changed structure in the strongly vacuolated cytoplasm. However, after 48 h recovery of the conditions, the swollen mitochondria are able to rebuild their membrane system [100].

Well-developed mitochondria are present in the rye mesophyll cells at 5°C, but in the cells of both cold-acclimated and cold-nonacclimated rye plants slowly frozen to -12°C, the mitochondrial cristae are strongly disorganized [121]. No significant differences in the respiration of the mitochondria in the extracellularly frozen cells of both acclimated and nonacclimated rye seedlings have been detected [122]. It can be concluded that mitochondria in situ retain normal function even after the cells have been killed by extracellular freezing. However, reports have shown that the mitochondria of rye leaf cells frozen in situ are much more susceptible to frost injury than the chloroplasts [121].

### 21.3.7 DICTYOSOMES

Abundant dictyosomes, usually composed of four to seven cisternae with numerous vesicles originating from their ends, are a common feature in the cells of different species not only during growth season but also during cold acclimation [85,91].

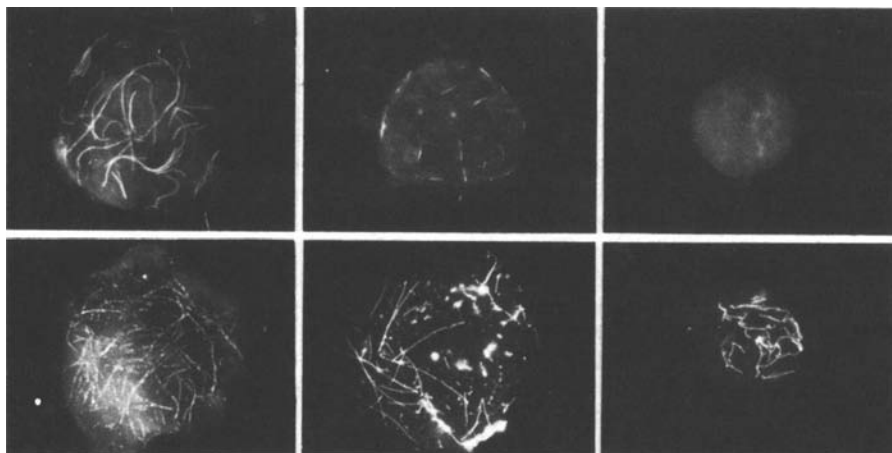
The presence of dictyosomes in poplar cortical cells in September is common. The number of dictyosomes decreases with fall in temperature in October and their level continues to decrease until the next spring [123]. In mulberry parenchyma cells, the dictyosomes secrete numerous vesicles and some of them are located beneath the plasmalemma during slow freezing at -5°C [88]. It may be possible that these secretory vesicles might participate in the formation of the multiplex lamellae that are very often found during slow freezing in mulberry cells.

In spite of the frozen state of tissues, dictyosomes in the cells of woody plants can be occasionally identified in their original form. There is evidence [85] that in mid November, when the cortical cells of apple survive freezing at a temperature of -20°C, the dictyosomes are still active and they produce vesicles. In late January, when the cells are hardy to a temperature of -30°C, dictyosomes can be observed, but they are not active. Similar alterations in the dictyosome ultrastructure and their localization in the cell have been observed in the cortical parenchyma cells from mulberry twigs [76], in the xylem ray parenchyma cells of *P. persica* [67], and in bark tissue of *R. pseudoacacia* [75].

### 21.3.8 CYTOSKELETON

The altered stability of the cytoskeletal elements at low temperatures has been recognized in different plant species. However, the lack of consensus regarding positive correlation between cold acclimation and the cold stability of microtubules still prevails [124,125]. Cold-induced depolymerization of microtubules at temperatures below 0°C has been observed; for example, in the cells of onion [43], cotton [126], spinach [124], garlic and winter wheat [127], and rye [125,128].

The effect of cold acclimation on cortical microtubule stability during freezing has been studied in cold-acclimated and cold-nonacclimated rye leaves [128]. The experiments have shown that unchanged microtubule arrays are still present in cold-acclimated leaf cells after -4°C temperature treatment. In contrast in the leaf cells of nonacclimated plants and in the root cells of both cold-acclimated and cold-nonacclimated plants the microtubules are shorter and less abundant. After a -10°C temperature treatment, the cortical microtubules are almost totally depolymerized in both types of root cells and in the leaf cells of nonacclimated plants, whereas cold-acclimated leaf cells constantly have abundant cortical microtubule arrays. Semiquantitative analyses of the cortical microtubules of protoplasts have confirmed the findings with intact leaf cells. These experiments have shown that the cortical microtubules of nonacclimated leaf cells are cold labile and cold acclimation induces cold-stable microtubules in leaf cells as well as in protoplasts (Figure 21.5) isolated from cold-acclimated leaves [128]. Hence, the cells need to have enough long cortical microtubules to keep their plasmalemma intact and responsive to the osmotic changes caused by subzero



**FIGURE 21.5** Responses of microtubules of isolated leaf protoplasts to freezing visualized by indirect immunofluorescence with anti- $\alpha$ -tubulin (1:100). Upper line (from left to the right) nonacclimated protoplasts: control protoplast, after freezing to  $-4^{\circ}\text{C}$  and after freezing to  $-10^{\circ}\text{C}$ . Lower line (from left to the right) cold acclimated protoplasts: control protoplast, after  $-4^{\circ}\text{C}$  treatment and after  $-10^{\circ}\text{C}$  treatment ( $\times 800$ ). (From Niki, T. et al. *Plant Cell Physiol.* 19, 139, 1978. With permission.)

temperatures. Under these stressful conditions, the microtubules may serve as a necessary support for the plasmalemma [128].

The crucial role of microtubules and/or microfilaments in the movement and reconstruction of the ER on the freezing has been reported in the cortical parenchyma cells of mulberry [88]. A contraction of the ER tubule (functional state) to a central rod (nonfunctional state) in the plasmodesmata during cold treatment is caused by changes in the actin–myosin filaments [129]. The partial disruption of actin filaments can accompany or promote freezing tolerance of carrot cell suspensions during preservation at extremely low temperatures [130].

## 21.4 MOLECULAR BIOLOGY AND BIOCHEMISTRY OF RESPONSE TO LOW TEMPERATURES

### 21.4.1 CELL MEMBRANES

Complex aspects of compositional alterations in the plasma membrane during cold acclimation have been reported. The concept of existence of microdomains in the plasma membrane has been introduced [131]. Microdomains were isolated from plasma membrane of *A. thaliana* as detergent-resistant membrane fraction (DRM). After cold acclimation, the proportion of free sterols, P-type  $\text{H}^+$ -ATPases, aquaporins, and endocytosis-related proteins increased in the DRM, and, conversely, tubulins, actins, and V-type  $\text{H}^+$ -ATPase subunits decreased [132]. This suggests that plasma membrane microdomains function as platforms of membrane transport, membrane trafficking, and cytoskeleton interaction [133].

There are still a limited number of known genes that are ultimately contributing to the increased stability of plasma membranes during low temperature stress. A low molecular weight plasma membrane protein from banana, encoded by *MpRCI*, was shown to directly modulate the physical properties of membrane upon stress, before an overall and more permanent response is initiated [134]. Closely related cold-induced genes/proteins were also identified in other plant species such as the WPI6 from wheat [135], BLST101 from barley [136], RCI2 from *Arabidopsis* [137], and OsLTi6 from rice [138].

In response to low temperatures, the plant membrane lipids have the tendency to change from gel to liquid-crystalline phase which is caused by increased lipid desaturation [139]. Modifications

of lipid unsaturation, controlled by key desaturases [140–142], lipases and invertases [143], account for a fraction of acquired freezing tolerance. Positive correlation between the unsaturated fatty acids and the electron transport activities, repairing PSII from damage from chilling, and integrity of the membranes has been reported [144]. However, interactions with other protective proteins such as dehydrins and chaperonins are necessary to fulfill a higher level of freezing tolerance [140]. In *Arabidopsis* [145] and rice [146], most of known genes for plasma membrane intrinsic proteins (the group of aquaporins) were repressed, while expression of the *AtPIP2;5* [145] and the abundance of both PIP1 and PIP2 proteins from maize [147] were induced by cold. Cold stress has also been shown to affect the post-translational modifications of proteins. Glycosylation and phosphorylation profiles of calreticulin, a key protein that regulates quality control of other proteins, are changed in rice leaf sheets by low temperatures [148].

#### 21.4.2 ENDOPLASMIC RETICULUM

The ER is considered to be the locus of lipid biosynthesis and is intimately concerned with the turnover of the membrane components. There is a fairly good correlation between the degree of unsaturation of the lipid phosphatidylglycerol (PG) and the known chill-sensitivity of many species, though it is not possible to generalize since such correlation was also absent in some species. It has been suggested that the expression of the stearyl-acyl carrier protein (ACP) desaturase gene in potato [140] and rapeseed [149] plants contributes to increase of cold tolerance in plants due to the increased desaturation of the fatty acids and thus a better membrane control of damage at the membrane level. In potato, the membrane lipids upon stress change their composition showing increase (by 5%–10%) in unsaturated fatty acids.

The cold-inducible WAP27 was found as an accumulate localized specifically in vesicular-form ER and also localized in dehydration-induced multiplex lamellae (MLP)-form ER [150]. It is hypothesized that in the ER it can stabilize the membrane in a manner similar to that of COR15am. Conversion of the ER to MPL and accumulation of WAP27 in the ER during winter have the specific effects of inhibiting or minimizing plasma membrane destabilization due to the close approach of membranes and consequently confer extremely high freezing tolerance to cortical parenchyma cells of mulberry tree [151].

Other cold-inducible proteins identified in the ER are the WAP27B that shows homology to LEA3 proteins [151] and WAP20 exhibiting homology to ER-localized small heat shock proteins (smHSPs) [150]. Both WAP27 and WAP20 are localized inside the ER vesicle and possibly play a significant role in the acquisition of freezing tolerance in cortical parenchyma cells of mulberry trees [151].

The ER structure reveals relative stability even in the absence (disorganization) of cytoskeletal structures during cold stress (see later) that manifests a certain organizational independence of this membranous organelle [152]. In addition, ER membranes might provide cytoskeletal monomers with the information important for their spatial organization during cold and also during subsequent recovery of actin filaments and microtubules at optimal temperatures.

#### 21.4.3 VACUOLE

In plant cells with acquired tolerance to freezing, the segmentation of the central vacuole is commonly known. This phenomenon was reported in winter rye [53], winter oilseed rape [153], as well as in moss cells *Physcomitrella patens* [82]. Restriction of vacuole volume associated with the appearance of numerous membrane-bound vesicles may be advantageous to frost-hardened cells. Interaction between water molecules and the polar head groups of membrane lipids might result in lowering of water potential in these compartments, which is an important factor for avoidance of excessive dehydration during formation of extracellular ice [153]. In *A. thaliana*, the reduction in vacuole volume and increase in cytoplasmic space in leaf cells induced cold acclimation. This is a

possible mechanism for accumulation of sucrose in a cytoplasm which contributes to protection of membranes from damages caused by dehydration and freezing [154]. Cryoprotectants like fructans are synthesized in vacuoles and they are probably transported to the apoplast by postsynthesis mechanisms which are probably induced by cold [155]. These findings indicate that segmentation of vacuoles might contribute to enhancement of freezing tolerance.

The vacuole also plays the role in calcium influx elicited by cold shock where the immediate rise in cytosolic free calcium concentration occurred. In this case, the vacuole served as an intracellular source of calcium [156].

The aquaporin GhTIP1;1 in cotton, that displays water channel activity and facilitates water transport through the vacuolar membrane, has been suggested to play a role in response to cold stress [157].

#### 21.4.4 CHLOROPLASTS

The photosynthetic activity declines in chilling-sensitive plants exposed to low temperatures as a consequence of decrease in the efficiencies of the both photosystems (PSI and PSII), the ATP synthase, and the stromal enzymes of the C3 carbon reduction cycle [158]. Although chilling can initiate the formation of reactive oxygen species (ROS) in membranes [159,160], peroxidation of lipids and finally leakage of cell content and loss of water [161], the chloroplast does not appear to be the primary target for oxidative damage at low temperatures [162]. In thylakoids, ROS are eliminated by photoprotective mechanisms [163], nonenzymatic antioxidants like ascorbic acid and  $\alpha$ -tocopherol [164,165], or by enzymes for example, superoxide dismutases (SOD) or peroxidases [166–168].

In the outer chloroplast membrane, a steroid binding protein lipocalin has been identified in wheat and *Arabidopsis* [169] that possibly contributes to increase of membrane fluidity at low temperature [170]. Further, enhanced expression of lipid desaturases like *fad7* [171] and *desC* [172] has resulted in increased number of thylakoids per granum in transgenic tobacco plants.

The cold regulated products of the *COR15a* gene of *A. thaliana* [173,174] and the *cor14b* from barley [175] in stroma probably protect the thylakoid membranes against freeze-induced damage. However, unlike the other cryoprotective proteins [176], COR15am appears to function by decreasing the tendency of membranes to form the lamella-to-hexagonal II phase, which leads to membrane damage during freezing [177]. Similarly, the *COR15b* gene of *A. thaliana*, the cold regulated *BN115*, *BN26*, and *BN19* genes of *Brassica napus* [178] and the *Cbcor15b* from *Capsella* [179], all apparent homologs of *COR15a*, encode for polypeptides that affect freezing tolerance.

Freezing tolerance is achieved by sequential stages of cold acclimation that is initiated independently by low/freezing temperatures and by short day length [180]. Phytochromes are responsible for detection of photoperiod. Modifications of the expression level of the phytochrome *phyA* gene from *Arabidopsis* have been shown to prevent cold acclimation or change in sensitivity to day length [181] by regulating levels of ABA and dehydrin (LEA group 2) [182]. However, these genes are regulated by a different mechanism when induced by extracellular freezing [180]. Plastid factors, like changes in plastoquinone redox state, affect nuclear genome activity [183] and elicit stress response mechanisms [184].

#### 21.4.5 NUCLEUS

The ability of plants to cold acclimate is a quantitative trait involving the action of many genes with small additive effects [185]. It has been proposed that perception occurs through low-temperature induced changes in membrane fluidity (rigidification), protein and nucleic acid conformation, specific metabolite- and/or redox status [186]. Activation of calcium channels or secondary signals such as ABA or ROS leads to cold-induced  $\text{Ca}^{2+}$  increase in the cytosol and calcium signal amplification,



and possibly phospholipid signaling are triggered [187]. It is proposed that in cabbage the gene *csp5* encoding a cold shock protein is a final link of signal-transmitting chain [188].

Metabolic profiling revealed that cold acclimation increases ~75% of the 434 metabolites detected in *Arabidopsis* [189]. In addition to their role as osmoprotectants, certain metabolites induced during cold acclimation might act as regulators of gene expression, for example, proline [190] or signal transducers for example, different soluble sugars [191].

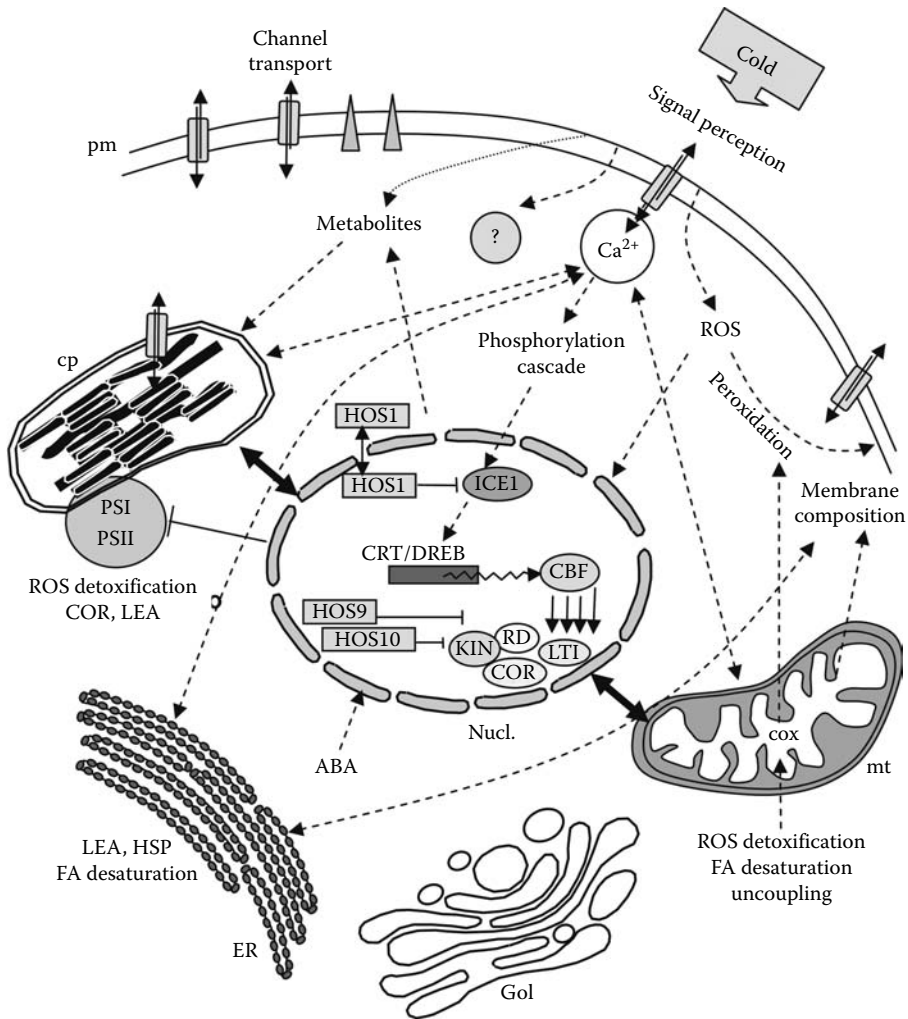
Transcriptome remodeling induced by cold represents up to ~20% of the genome [192,193]. In cold-acclimated winter wheat at slowly freezing conditions the number of upregulated genes reached a ratio of 9:1 with those of the downregulated [194]. In these analyses, cold regulated (COR), dehydration responsive, and ice re-crystallization inhibition (IRI) genes were upregulated and photosynthesis and respiration-related genes were repressed upon cold stress. Many COR genes have in their promoters one or several copies of the dehydration responsive element/C-repeat cis-element (*DRE/CRT*) that is recognized by transcription factors known as CRT-binding factors (CBFs) or DRE-binding factors (DREBs) [195,196]. The CBFs are expressed early and transiently by cold stress and have been identified in many plant species (for review see [197]). Their expression levels are sensitively regulated positively by, for example, cold-activated protein kinases, mitogen activated protein (MAP) kinase cascade [198], ABA, circadian clock [199], by ubiquitination-mediated proteosomal protein degradation of the *ICE1* (Inducer of CBF Expression 1) [200] as well as by calmodulin binding transcription activator (CAMTA) [201]. On the other hand, CBFs are negatively regulated by the *HOS1* (high expression of osmotically responsive gene 1) that targets *ICE1* for proteosomal degradation [202–204]. The *HOS1* gene is constitutively expressed but gets drastically downregulated within 10 min of cold stress. Posttranscriptional RNA processing and nucleocytoplasmic transport have been shown to play an important role in cold acclimation. Different RNA chaperones, RNA helicases, zinc-finger-containing glycine rich RNA binding proteins are upregulated by cold stress and contribute to freezing tolerance [205].

CBFs activate a core set of robustly COR “downstream” plant genes including genes known as *COR*, *KIN* (cold-induced), *LT1* (low-temperature-induced), or *RD* (responsive to dehydration) genes. Products of these genes include different dehydration-responsive proteins, enzymes required for biosynthesis of osmoprotectants and antifreeze proteins (AFPs). The AFPs (products of, e.g., *CHT9* and *CHT46* from winter rye, *sthp*-64 from *Solanum dulcamara*, *PsAFP* from *Populus suaveolens*) belong to effective cryoprotectants accumulated only during cold acclimation [206]. They possess multiple, hydrophilic ice-binding domains and appear to function as inhibitors of ice recrystallization and ice nucleation.

Accumulation of the downstream gene products acts to minimize the injury caused by dehydration, or would limit ice crystal growth. For example, many COR or LEA proteins including dehydrins function as membrane stabilizers and thus enhance cold tolerance in plants [185]. Overall, cold acclimation results in protection and stabilization of the integrity of membranes, activating antioxidative mechanisms, accumulation of soluble sugars and other cryoprotectants, and activation of repair mechanisms (e.g., inducing chaperones) (Figure 21.6).

Although the CBF cold regulatory pathway is commonly thought to play a key role in cold-responsive mechanisms, CBFs regulate only ~12% of the cold-responsive genes [207]. There have been several CBF-independent regulons identified that are critical for cold acclimation. For example, mutations in the *HOS9*, *HOS10* [208] or *ESK1* [209] genes result in stronger or shifted induction of several CBF-target genes and lead to altered freezing tolerance. However, they exert no effects on the expression of CBFs. For a more detailed view on the network of COR genes in cold acclimation we refer the reader to reviews reported by [186,197,210]. Interestingly, the data indicate that the genetic and molecular basis of subzero acclimation is probably different from that of cold acclimation [211].

Proteomic analyses identified changes in nuclear proteome in response to cold stress. From all identified proteins 54 were up- or down-regulated in *Arabidopsis* [212] and sixty was upregulated in rice [213]. More recently, increased evidence points toward the existence of plant nuclear proteins



**FIGURE 21.6** Molecular interactions in the cell under low temperatures. After signal perception, the composition of the membrane (pm) is altered and calcium ( $\text{Ca}^{2+}$ ), ABA or reactive oxygen species (ROS) mediate signal amplification. In the nucleus (nucl.) the transcription of many genes is altered, largely repressed. Many genes containing in their promoters DRE/CTR elements are regulated by CTR-binding factors (SBFs), DRE-binding factors (DREBs), ICE (inducer of CBF expression) activator or by HOS (high expression of osmotically active genes) negative regulators. CBFs affect different cold regulated—(COR), cold induced—(KIN), low-temperature induced—(LTI) or responsive-to-dehydration (RD) genes that leads to production of specific protective proteins to avoid chilling damages. These include among others different heat shock proteins (HSPs), late embryogenesis abundant proteins (LEAs), and ROS detoxification proteins. In mitochondria (mt) the level of fatty acid (FA) saturation is altered and activity of cytochrome c oxidase (cox) is negatively affected. In chloroplast (cp), the activity of the photosystems (PSI and PSII) is repressed. Water status is carefully controlled, e.g., by LEAs or channel transport.

that are also targeted to other organelles—either mitochondria or plastids [214]. Most of such dual-targeted proteins identified so far are transcription factors that play a role in the genome structure maintenance by binding to telomeric DNA. Though the knowledge on their distribution patterns and distribution mechanisms is still rather limited, proteins with dual locations are excellent candidates for signal transduction and coordination of gene expression between the nucleus and the organelles involved in (cold) stress perception like chloroplasts and mitochondria [214].

#### 21.4.6 MITOCHONDRIA

Mitochondria have been found to be the main cellular compartment affected by chilling treatment. Some of the most notable effects of cold on mitochondria are reversible uncoupling [215], decrease in protein import [216], and accumulation of stress related proteins. These include, for example, heat shock proteins such as the HSP22 [217], late embryogenesis abundant (LEA) proteins [218], peroxidases [219], and catalases [220–222]. Under low-temperature condition the involvement of alternative oxidase (AOX) [223] has been suggested to play role in *Arabidopsis* shoot acclimation [224], and cold acclimation and freezing tolerance in wheat [225]. Plant mitochondria possess alternative enzymes enabling oxidizing of both external NAD(P)H and internal NAD(P)H independently of complex I [226], providing additional flexibility to the mitochondrial metabolism during cold stress [227,228].

Mitochondria from the cold-tolerant population of maize genotypes revealed a higher percentage of 18-carbon unsaturated fatty acids when compared to the sensitive ones that has been associated with the physiological need to maintain membrane fluidity at low temperatures [230]. The level of 18:1 and 18:3 unsaturated fatty acids can control the activity of cytochrome c oxidase (COX) [229] that decreases in the inner mitochondrial membrane at chilling temperatures and causes enhanced production of H<sub>2</sub>O<sub>2</sub> and subsequent lipid peroxidation in the membranes [219]. Efficient scavenging of H<sub>2</sub>O<sub>2</sub> by antioxidant enzymes such as superoxide dismutase, catalase, and peroxidases [219,220,222,231] are necessary to prevent the irreversible damage of mitochondrial membrane components [231].

In addition, the functional state of mitochondria affects expression of nuclear genes [232] leading to further responses to low temperatures.

#### 21.4.7 CYTOSKELETON

Inside the cells, the cytoskeleton can serve as a template where components transducing extracellular signals interact. Recently, the novel protein HOS1, a putative E3 ubiquitin ligase controlling degradation, was shown to reside in the cytoplasm at normal growth temperatures but accumulate in the nucleus in response to low temperatures [203,204]. The HOS1 is then likely to contribute to the communication between the cytoplasm and the nucleus under cold conditions.

In addition, cold acclimation induced receptor kinase from wheat [233] and a large number of regulatory proteins [234] have been found to interact with microtubules supporting the view that microtubules play a central role in cold signaling and cold acclimation [235]. Despite the fact that cold stress conditions strongly inhibit protein synthesis *in vivo*, the levels of actin and tubulin in cytosolic and sedimentable protein fractions do not change under low temperatures (neither degradation of particular mRNAs occur) [152]. Cold acclimation caused the aggregation of microtubules, which is demonstrated by increased fluorescence and stability of microfilaments in root cells of *Triticum aestivum* [236]. Further, appearance of cold-stable microtubule was accompanied by reduced abundance of type TUA ½  $\alpha$ -tubulin isotypes [41] as well as they were fine and transverse strands [237]. Cold-resistance of microtubules is explained by amino acid substitution in sequences of tubulin molecules [238]. Microtubules are not only the target of cold stress, but they also seem to participate in cold sensing itself, triggering a chain of events that results in increased cold hardiness [239].

#### 21.4.8 CELL WALL

The cell wall acts as a barrier against propagation of extracellular ice. This is documented by experiments with plasma membrane disrupted cells by repeated freezing and thawing. In chilling-sensitive plant cells intracellular freezing occurred, whereas in chilling-resistant ones, the response to freezing was only by extracellular freezing [240]. Different pore size of the cell walls

of chilling-sensitive and of chilling-resistant plants is believed to reflect on long-term cold acclimation due to the evolutionary adaptation of plants to cold climates [241]. The process of cold acclimation also includes increased cell tension accompanied by ability of cell walls to undergo deformation. It was demonstrated that in suspension-cultured cells of grape and apple the cold acclimation was the result of increase in the cell-wall strength and a decrease in the cell wall pores [242]. Mechanical properties of cell wall are influenced by temperature dependent modifications in pectin content and their methyl esterification degree which increased its resistance to freezing of oil-seed rape plants [243] and chicory root [244]. The mechanism of deep supercooling in xylem ray parenchyma cells in hardwood species was proposed to allow adaptation to freezing [245].

## 21.5 CONCLUSION

From the presented results it is obvious that low-temperature stress considerably affects the structure of plant cells. The structural response of the cells is variable and is determined by external (strength and duration of stress) and internal (plant species, ontogenetic phase of the plant, type of the tissue, and genetically determined level of resistance) factors. Therefore, it is difficult to decide which cell compartment plays the primary or the most important role in cell responses to both chilling and freezing stresses.

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# 22 Effects of UV-B Radiation on Plants: Molecular Mechanisms Involved in UV-B Responses

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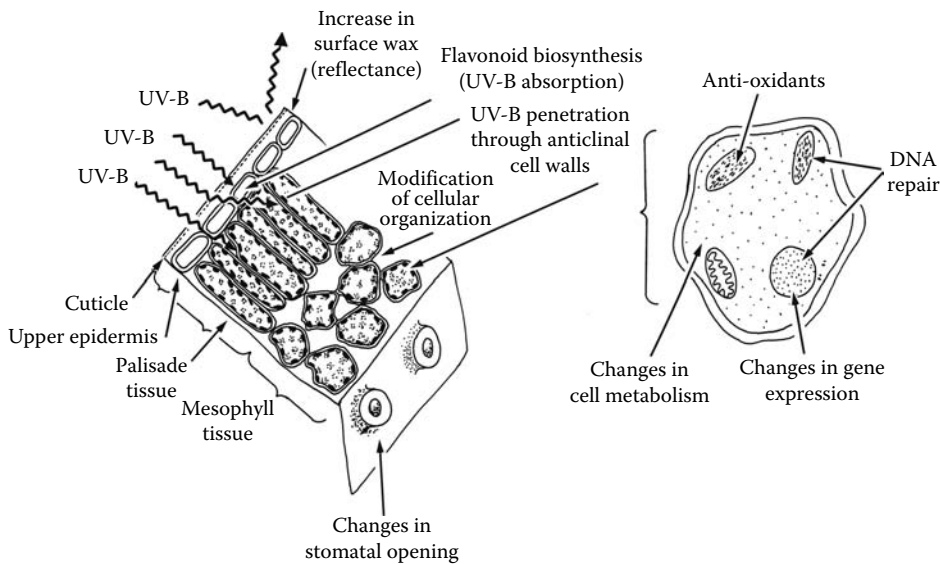
## 22.1 INTRODUCTION

Ultraviolet (UV) radiation is divided into UV-A (320–380 nm), UV-B (280–320 nm), and UV-C (below 280 nm). UV-C is essentially a lethal radiation and is excluded from reaching the earth’s surface by the ozone layer. UV-B, however, is only partially excluded (<290 nm) and is an extremely energetic radiation capable of causing substantial changes to plant form and function. These changes include:

- Effects upon plant development and morphology
- Effects vary between species and within varieties of the same species
- Changes in gene expression, both up-regulation and down-regulation
- Specific effects upon photomorphogenesis at low fluence, potentially through photoreceptor(s)
- Effects upon cellular metabolism including, metabolic channeling, metabolic feedback and changes in oxidation status
- Changes to primary and secondary metabolism
- Potential impact on the competitive ecology between plant species and with other organisms

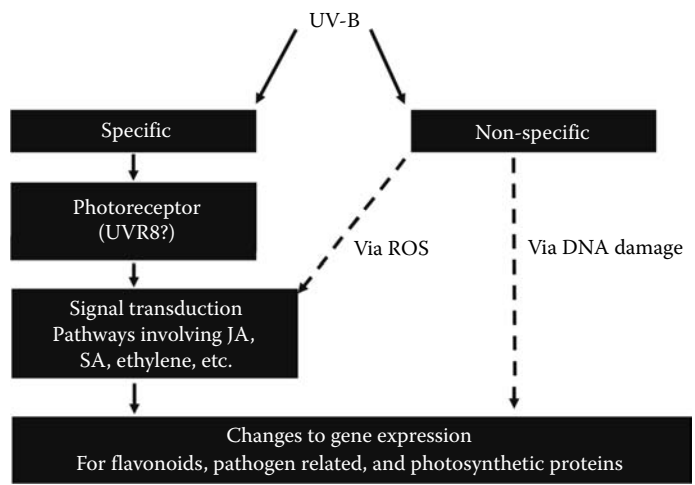
UV-A is thought to be less harmful, but can still influence plant growth and development. In addition to UV radiation, visible radiation covers the electromagnetic spectrum between 380–700 nm; blue to red and referred to as photosynthetically active radiation (PAR). Plants can also detect wavelengths in the far-red region and regulate photomorphogenesis (e.g., phytochrome).

UV-B has generally been thought of only as a stress factor for plants, which stimulates a wide range of defense mechanisms that include synthesis of protective pigments, DNA repair, and the production of antioxidants (Figure 22.1). However, a more considered perspective of UV-B is now being discussed in terms of ecological and environmental impact at a variety of trophic levels (Day, 2001; Rozema et al., 1997). Thus, UV-B radiation is part of the natural environment and may both



**FIGURE 22.1** Protective mechanisms against UV-B radiation. Illustration of a leaf cross section and an enlarged mesophyll cell. (From Jordan, B.R., The effects of ultraviolet-B radiation on plants: A molecular perspective, in *Advances in Botanical Research*, ed. J.A. Callow, vol. 22, Academic Press Ltd, New York, 97–162, 1996, Fig. 5. With permission.)

induce stress and act as a signal/regulator for plant photomorphogenesis (Brosché and Strid, 2003; Frohnmeier and Staiger, 2003; Hectors et al., 2007; Jenkins and Brown, 2007). To some degree, this is no way different to high PAR causing stress through photo-damage to the photosynthetic apparatus or specific wavelengths of red or blue light regulating growth and development. However, a major issue regarding the role of UV-B is the specificity of the response. Thus, UV-B radiation is absorbed by a very wide range of biological molecules, DNA, proteins, lipids, flavonoids, etc. This nonspecific absorption of UV-B wavelengths is very hard to equate with a defined mechanism of action. The alternative, however, is that a specific photoreceptor is involved to perceive the UV-B radiation and induce a response through one or more signal transduction pathways to change gene expression (Figure 22.2). At this point in time, no photoreceptor has been convincingly demonstrated



**FIGURE 22.2** UV-B-induced changes in gene expression. Schematic to illustrate the direct and indirect pathways leading to UV-B responses.



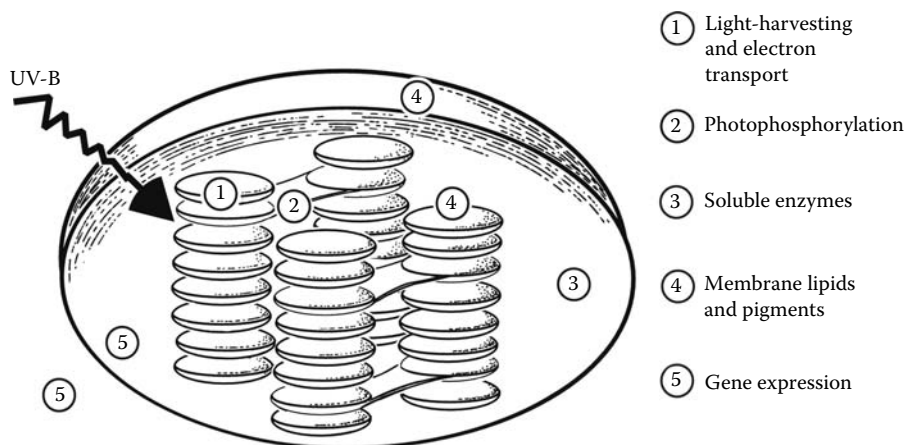
to function in this role. However, progress has been made recently to address this critical issue and a putative candidate has been identified (Jenkins, 2009). Furthermore, a greater understanding of the signal transduction pathways linking UV perception to gene expression is being determined (Jenkins, 2009; Jenkins and Brown, 2007; Jordan, 2002; Mackerness, 2000). These pathways may in themselves separate between more general stress-related responses and those that are specific to a photomorphogenetic response.

A number of factors are very important when we consider the potential effects of UV-B radiation on plant growth and development. These factors have been very well described and reviewed (Jansen et al., 1998; Jordan, 1996, 2002; Ulm and Nagy, 2005), but to a large extent the underlying molecular mechanisms remain undefined.

A summary of these responses is as follows:

- Perception of the light environment
- Penetration of UV-B into the tissue
- Damage to DNA and efficiency of repair mechanisms
- Signal transduction cross-linked to other abiotic and biotic stress factors
- Developmental stage of the tissue
- Tissue-specific regulation
- Strong interaction with other environmental parameters
- Differential response of gene family members
- Genotype response: productivity or stress protection

The most obvious factor that will determine the response is the penetration of UV-B into the tissues (Figure 22.1). This penetration is species dependant and takes place through the anticlinal cell walls of the epidermal layer (Day, 1993; Jordan, 1996 and references therein). However, if a specific photoreceptor is present, for instance, in the epidermal layer, penetration of UV-B may not be a prerequisite for a response. Another factor to consider is that the UV-B-induced response is modified by other environmental parameters. For instance, high visible light delivered at the same time as the UV-B will reduce the damaging effects compared to low light (Jordan et al. 1994; Mackerness et al., 1996, 1998 and see below for further discussion). The mechanism of this response is unknown, but is not acclimation or adaption, which also takes place (see Jordan, 1996 and references therein). Water availability, temperature, and many other abiotic stress factors can also modify the UV-B response. In a similar manner, there is a strong interaction of UV-induced responses and those that are brought about by biotic stress, such as herbivory or pathogens (Izaguirre et al., 2003; Lindroth et al., 2000). Another significant influence on the UV-B response is the stage of plant development at which the exposure takes place (Mackerness et al., 1998). As a general rule, younger plants do not seem to be as affected as do older plants (John et al., 2001; Lytvyn et al., 2009). UV-B radiation appears to induce accelerated senescence of the older tissues, under conditions that do not cause changes in young plants. It is also well known that the response to UV-B is different between plant families and also varieties of the same family (Hofmann et al., 2000a; Hofmann et al., 2000b). A final factor to consider is the changes in chemical composition that take place in response to UV-B exposure. The most characteristic change is both a quantitative and qualitative change in phenolic compounds (Bornman et al., 1997; Jordan, 2004). These compounds increase substantially and act as “sun-screens” to protect the plant. In addition, UV-B frequently changes their overall chemical structure to enhance the protective potential. For instance, conversion between similar molecules such as a preference for quercetin compared to kaempferol or luteolin compared to apigenin (Markham et al., 1998; Ryan et al., 1997). These, or similar changes, can provide a protective benefit by adding to an overall increase in antioxidant potential within the tissue. This alteration in the chemical composition as a defense strategy requires an overall change in cellular metabolism that could include, for example, changes to photosynthesis. Certainly, the chloroplast is a very susceptible organelle as a center of photosynthesis, nitrogen assimilation, and lipid metabolism (Figure 22.3). Any change



**FIGURE 22.3** Potential sites of UV-B damage to chloroplasts. (From Jordan, B.R., The effects of ultraviolet-B radiation on plants: A molecular perspective, in *Advances in Botanical Research*, ed. J.A. Callow, vol. 22, Academic Press Ltd, New York, 97–162, 1996, Fig. 6. With permission.)

from primary metabolism to secondary metabolism caused by UV-B is in itself a substantial issue for crop productivity (Jordan, 2004), particularly in a changing climate scenario.

It is now more than 10 years since the review of Mackerness and Jordan (1999) and substantial progress has been made in our understanding. To cover the advances that have taken place, the reader is advised to read a number of excellent reviews (Jenkins, 2009 and references therein; Jenkins and Brown, 2007; Jordan, 2002; Mackerness, 2000; Ulm and Nagy, 2005). The present chapter will focus on updating the understanding of UV-B perception, signal transduction, and changes in gene expression. While understanding the molecular mechanisms of UV-B response is very important, it is also important to put these in the context of a crop situation. As part of this review therefore, viticulture will be used as a “real” example of the impact of UV-B on fruit development.

## 22.2 UV-B PERCEPTION

Great progress has been made in the identification and characterization of photoreceptors such as phytochrome, cryptochrome, and phototropin (Whitelam and Halliday, 2007). In contrast, a defined photoreceptor for the UV part of the electromagnetic spectrum has not been identified. This is, in part, due to the very wide range of molecules that absorb UV wavelengths. Suitable UV-induced phenotypic changes are also more difficult to characterize as they are specific only to a UV photoreceptor. It is, however, likely that UV photoperception is not mediated through other known photoreceptors as mutants lacking photoreceptors, such as phytochrome, show UV responses (Jenkins and Brown, 2007). DNA has previously been raised as a possible candidate for UV-B photoperception. This is unlikely, however, as the experimental evidence does not support a specific photoreceptor role for DNA. For instance, photomorphogenic responses take place at fluence rates below detectable DNA damage, and DNA damage repair mutants do not show an enhanced response to UV-B. Action spectra also indicate a photoreceptor with maximum absorbance between 295 and 300nm and this suggests the involvement of flavin-like compounds as the chromophore (Ballaré et al., 1995; Ensminger, 1993). DNA damage through UV-B, however, may play a role in controlling cell-cycle regulation (Culligan et al., 2004). Thus, mutants lacking ATR (ataxia telangiectasia mutated and RAD3 related) protein kinase are sensitive to UV-B. ATR is important in controlling cell cycle progression when DNA replication is prevented. Recently, some further insight has been gained into the perception mechanism involved in UV responses. Shinkle et al. (2004, 2005) studied hypocotyl growth in cucumber and a number of other dicotyledonous seedlings exposed to different wavelengths within the UV region. They found that all wavelengths induced the transient inhibition of elongation, but varied in other responses in a

wavelength-dependant manner. For example, wavelengths predominantly below 300nm induced the inhibition of elongation within 20min of irradiation and were persistent for a minimum of 24h. This contrasted with longer wavelength treatments (only 8% between 290 and 300nm) in which the lag phase was 1–2h and the inhibition lasted only 2–3h. UV-A was different again, with reductions in elongation rates of 6–9h followed by a continued decline for the next 15–18h. These results indicate that different photoperception mechanisms are induced by short-wavelength UV-B, longer-wavelength UV-B, and UV-A. This recognition at the physiological level of different perception mechanisms has been complemented by several studies using molecular approaches (Kalbina et al., 2008; Ulm et al., 2004, 2005). Ulm et al., 2004 used cut off filters to create four regions within the UV-B spectra. After exposure of *Arabidopsis* seedlings to 15min of irradiation they analyzed 24,000 genes using oligonucleotide microarrays. These experiments strongly indicated that at least two UV-B perception mechanisms exist (280–300nm and 300–320nm). In addition, the results separated the responses from other known photoreceptors and signal transduction caused by DNA damage. Kalbina et al., 2008 came to a similar conclusion after studying four genes. Their studies suggested that the genes were regulated by either a chromophore absorbing at or above 300nm and one absorbing between 280 and 290nm. The authors suggested that a higher-wavelength chromophore may be involved with the UVR8/HY5 signaling pathway (see below). Another important area of photoreceptor and signal transduction biochemistry is the role of phosphorylation (Lillo et al., 2007; Watson, 2000). Both photoreceptors themselves and components of the signal pathway may act as kinases or substrates for phosphorylation. To date, there is no strong evidence for phosphorylation being involved in the UV-B responses, but indirect evidence suggests that this is an area for further investigation (Jenkins, 2009; Jenkins and Brown, 2007).

It seems apparent that more than one perception mechanism and overlapping signal transduction pathways are involved in UV-B responses. Progress has been made at unraveling these complex interactions, but there is still a long way to go. Confirmation of a UV-B photoreceptor and the molecular mechanism involved in its activation remains the “grail” to make a quantum advancement in UV-B research.

## 22.3 SIGNAL TRANSDUCTION PATHWAYS

As mentioned above, there is considerable discussion on the role of UV-B as a regulator of photomorphogenic events in addition to inducing a stress response. This is particularly reflected in the area of UV perception, signal transduction and gene expression. UV-B has been shown to both up-regulate and down-regulate gene expression (Jordan, 1996, 2002; Mackerness, 2000). The regulation, however, can be separated into low-fluence and high-fluence responses (Brosché and Strid, 2002): the higher fluence being associated with a stress-induced response, and the response caused by low-fluence UV-B being thought of as a more specific photomorphogenic induction. This is a reasonable working model, but under natural conditions, the response may not be quite so simple (see below). The high-fluence response seems to work through a number of signal transduction pathways that are also caused by pathogens, herbivory, wounding, etc. An early event in this process is the formation of reactive oxygen species (ROS), leading to changes in gene expression. The UV-B induction of signal transduction through ROS can be inhibited by antioxidants (Surplus et al., 1998) and scavengers of superoxide (Mackerness et al., 2001). ROS formation is complex given the multitude of potential sources, but an obvious candidate is lipid breakdown, particularly in chloroplasts. Chloroplast membranes are rich in polyunsaturated fatty acids and consequently very susceptible to UV-B damage. Furthermore, a chloroplast-induced signal is known to regulate gene expression (Nott et al., 2006; Taylor, 1989). However, ROS generation in chloroplasts does not seem to be involved in UV-B induced signal transduction (Jordan et al., 1998). Superoxide itself has been shown to be formed by NADPH oxidase in defense signaling (Apel and Hirt, 2004). A similar mechanism has been suggested in response to UV-B (Mackerness et al., 2001). Although ROS is involved in the UV-B regulation of a number of

specific genes (up-regulation of PR-1,-2,-5, PDF1.2 and *LHCB*, *RBCL*, *RBCS* and *PSBA*), it does not regulate CHS (Brosché and Strid, 2002; Jenkins, 2009). This is particularly interesting and indicates a different signaling pathway that may relate more specifically to the photomorphogenic response (see below). The UV-B signaling pathway that includes ROS also involves defense pathway intermediates. These include jasmonate, salicylate, and ethylene. In a series of experiments using a range of approaches (transgenic and mutant plants, inhibitors, etc.), Jordan's group deduced UV-B signal transduction intermediates from ROS through to changes in gene expression for PR genes, photosynthetic proteins, and flavonoid biosynthesis (reviewed in Jordan, 2002; Mackerness et al., 1999a and 1999b, 2001; Surplus et al., 1998). These studies also demonstrated a crossover between pathways. For instance, ethylene was not only involved in the up-regulation of PDF1.2, but also had a role in a salicylate mediated regulation of PR-1. This differs from pathogen-induce up-regulation of PR-1, which does not involve ethylene.

The high UV-B fluence-induced pathway becomes more complex as a biotic stress, such as a pathogen, becomes involved. Under these conditions, it would seem that there is a hierarchy of responses with the pathogen response being most dominant (Logemann and Halbrock, 2002). In addition to biotic stress, we must also consider the influence of other environmental parameters on the UV-B-induced response. Thus, it is well known that UV-B responses are changed by other environmental factors such as light, temperature, and water availability (Jordan, 1996). These components are both part of the natural growth environment for plants and at times can be additional abiotic stress factors. Very little consideration seems to be given as to the interaction of signal transduction pathways responding to abiotic stress. This seems a rather glaring gap in our knowledge when these strong environmental interactions are well known. A particularly interesting interaction is light. A number of studies have shown that UV-B does not act through well-characterized photoreceptors such as phytochrome or cryptochrome (Boccalandro et al., 2001; Jenkins and Brown, 2007 and references therein). However, there is a very clear interaction between PAR intensity and UV-B-induced changes in gene expression (Jordan et al., 1992, 1994; Mackerness et al., 1996). This interaction is not thought to be due to photorepair mechanisms such as photolyase, but rather as depending on photosynthesis itself; particularly electron transport and photophosphorylation (Mackerness et al., 1996). If this is the case, then what type of signal can override the UV-B-induced signal transduction pathway? A sugar signal from the chloroplast would seem a logical contender. However, experiments with *Arabidopsis* indicate that this is not the case (Mackerness et al., 1997). Another aspect of how the signal pathways are regulated is in response to development and acclimation. Many studies show that plants at different stages in development respond differently to UV-B. For instance, UV-B severely inhibits gene expression for photosynthetic proteins in mature leaves of pea seedlings, but corresponding genes in leaf buds remain active (Mackerness et al., 1998). In white grape berries, UV-B induced formation of pigmentation takes place only after a particular stage of development, veraison (see below). Furthermore, during de-etiolation, genes for photosynthetic proteins continue to be expressed in the presence of high UV-B irradiation (Jordan et al., 1994). In a similar manner, many studies show that high light prior to UV-B exposure protects the plant. Is this just a physical or biochemical protection or is signal transduction mechanism involved and a "memory" imprinted into the system?

As mentioned previously, it has proved difficult to isolate a specific component of the UV-B signal transduction pathway. However, recently a specific signaling component, UVR8, has been isolated (Brown et al., 2005 and reviewed in Jenkins, 2009; Jenkins and Brown, 2005; Kliebenstein et al., 2002). Using a CHS promoter: luciferase construct mutagenised *Arabidopsis* were exposed to UV-B and those lacking CHS expression identified. Over 50,000 EMS mutagenised plants were screened for the noninduction of CHS and four independent mutants selected. These four independent mutants were then shown to be allelic to *uvr8-1* isolated by Kliebenstein et al. (2002). Further studies confirmed the specific role of UVR8 and the potential for it to act through Elongated Hypocotyl5: HY5 (Jenkins and Brown, 2005).

Although UVR8 has sequence similarities to RCC1 (Regulator of Chromatin Condensation1), it is unlikely to be a functional homologue. However, UVR8 shares some similarities in that it associates

with chromatin in the nucleus as does RCC1 (Cloix and Jenkins, 2008; Jenkins and Brown, 2007). The distribution of UVR8 is not only localized in the nucleus, but also present in the cytoplasm. This is unlike all other members of the RCC1 family. Further studies (Kaiserli and Jenkins, 2007) have clearly shown that UVR8 redistributes to the nucleus. This redistribution is UV-B specific, takes place at low fluence, and is relatively rapid (within 5 min). A 23-amino acid n-terminal region is required for the transfer of UVR8 to the nucleus, but this region does not contain a nuclear localization signal. This implies that UVR8 needs another reaction partner to facilitate translocation into the nucleus, possibly analogous to the mechanism involved in the transfer of phytochrome A into the nucleus. The relocalization of UVR8 leads to the activation of HY5, which is a transcription factor linked to the activation of UV-B-induced genes. However, there is still a requirement for UV-B to cause the expression of HY5, suggesting that UV-B both causes the relocation of UVR8 into the nucleus and activates it within the nucleus. HY5 itself is regulated by the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) protein. COP1 represses photomorphogenesis by targeting the proteolytic degradation of HY5 mediated by the ubiquitin/proteasome pathway and the amount of HY5 is inversely proportional to the abundance of COP1 in the nucleus (Feng and Deng, 2007). Microarray analysis showed that 75% of genes normally induced by a photomorphogenic fluence of UV-B gave reduced expression in *cop1* mutants, including HY5 and CHS (Oravec et al., 2006).

A number of approaches indicated that UVR8 is quite specific in its response to UV-B and not dependant on other photoreceptors or non-light stimulants, such as sugar, temperature, etc. Using microarray technology to study gene expression in *uvr8* and wild-type plants, UVR8 was shown to induce a number of genes (a minimum of 72 with a 5% estimate of false positives) regulated at a photomorphogenic level (Brown et al., 2005). Amongst these genes were those for flavonoid and alkaloid biosynthesis, photoprotection, such as photolyase and ELIP in addition to a range of transcription factors. Despite the fact that the induction was primarily to promote photomorphogenesis, a large number of genes expressed still play a major role in protecting the plants from UV-B. UVR8 also seems to play a role in other aspects of plant responses to UV-B, including a role in leaf development (Wargent et al., 2009). UV-B reduces leaf area in a range of plant species probably through a reduction in leaf expansion and cell division. UVR8 does not seem to play a role in epidermal cell division, but does contribute to the regulation of epidermal cell expansion. This role in leaf expansion is likely to take place through UVR8-regulating endopolyploidy. In addition to leaf development, UVR8 is required to maintain the relative density of stomatal pores on exposure to UV-B.

## 22.4 GENE ACTIVATION AND EXPRESSION

It is apparent that a number of parallel and interacting signals may be directed from the initial UV-B perception and ultimately interact with a variety of different genes. These genes, however, are also being regulated by a range of other signals induced by biotic and abiotic stress factors. If these multiple and diverse signals do converge on the same gene promoter, they may then elicit a different response. One good example of such a mechanism is the ACE cis-acting element (elements containing ACGT that recognize common plant regulatory factors) that responds positively to UV and negatively to a pathogen-derived elicitor (Logemann and Halhbrock, 2002). The elicitor response overrides that of the UV signal and suggests that there is a hierarchy or responses mediated through the ACE-element subsequent to receiving convergent signals. The previous review of Mackerness and Jordan (1999) discussed in some detail the factors that influence gene expression. For the most part, the studies being reviewed were on small numbers of individual genes, or gene families. The major change in approach since this time is the application of microarray technology to look at large numbers of genes (Brown et al., 2005; Casati and Walbot, 2003, 2004; Casati et al., 2006; Ulm et al., 2004). These approaches have confirmed many of the basic findings of the previous small-scale experiments. For instance, genes for photosynthetic proteins, such as *RuBp* carboxylase and *Lhcb* are down-regulated while those for UV-protecting pigments are up-regulated. The major benefit from the microarray approach is that the majority of gene changes are captured. In a series of studies, the team of Virginia

Walbot has investigated the effect of UV-B radiation on maize (Casati and Walbot, 2003, 2004; Casati et al., 2006). Using near isogenic lines of field-grown maize, 2500 expression sequence tags (ESTs) were analyzed and 355 showed a response to UV-B. Approximately two-thirds of the ESTs were assigned a function. UV-B increased the expression of stress-related genes, antioxidants, protective pigments, etc., and ribosomal protein genes. The down-regulated genes included those for photosynthetic proteins as previously found in controlled environment studies. In near isogenic lines with reduced protective pigments, the UV-B-induced response was more pronounced as expected given the important protective role of these pigments. A further study by Casati et al. (2006) was carried out on high-altitude landraces of maize and compared to low-altitude lines. The most significant finding was that several genes associated with Chromatin remodeling were differentially expressed before UV-B and after UV-B treatments. Using RNAi transgenic plants to lower the expression of four of these genes created plants that were hypersensitive to UV-B. These results suggest that chromatin remodeling is an important component of UV-B protection. It is also particularly noteworthy that mutants in the *Arabidopsis* *uvr8-1* gene, which is likely to be involved in chromatin modification, are hypersensitive to UV-B and resemble the molecular phenotypes of the RNAi-modified maize. Ulm et al. (2004) used microarray to study the response to UV-B in *Arabidopsis*. They found that HY5 was induced by UV-B and in subsequent experiments showed that it was required for the expression of a number of other UV-B-induced genes. Furthermore, it was shown that UVR8 controls HY5 expression. To extend these studies, chromatin immunoprecipitation has been used to locate UVR8 in relation to transcription factors (Jenkins, 2009; Brown et al., 2005). These experiments showed that a GFP-UVR8 associated with a chromatin region containing the HY5 promoter sequence. Although our understanding of the role of chromatin is far from clear, the developments in this area of study are promising in terms of our overall understanding of UV-B responses.

## 22.5 GRAPE DEVELOPMENT: A REAL-LIFE EXAMPLE OF UV-B RESPONSES

There are relatively limited examples of UV-B responses in the natural environment, particularly relating to commercial crops. However, one commercial crop, grape, is frequently subjected to UV-B responses through management practice (see below), and this could have an influence on the final quality of the fruit. Specifically, the response to UV-B is particularly significant because many potential UV-B-induced changes alter chemical compositions that are important in the wine-making process. For instance, UV-B can change the levels and types of flavonoids, which can influence the bitterness and mouthfeel of the wine. In many parts of the Southern hemisphere where grapes are grown for wine production (e.g., New Zealand, Australia, and Chile), the vines are exposed to relatively high levels of UV-B. In New Zealand for instance, UV-B levels are 40%–50% higher than those of an equivalent latitude in the Northern hemisphere, exacerbated by the clear unpolluted air and high overall light intensity. This is particularly significant when the management practice of leaf removal is considered. Leaf removal around the fruit zone is frequently used to reduce humidity and hence reduce disease pressure on the grape clusters. Thus, not only are the remaining canopy leaves exposed to high UV-B, but also the berries themselves may be directly exposed. There is also a grape-developmental stage called veraison at which time the berries soften rapidly over a few days (Kennedy, 2002). This transition seems a particularly important developmental switch in respect to responsiveness to UV-B. There have been relatively few detailed studies of the changes that occur in grapes in response to UV-B considering the potential importance (see examples: Keller and Torres-Martinez, 2004; Kolb et al., 2001; Lenk et al., 2007; Núñez-Olivera et al., 2006; Schultz, 2000; Schultz et al., 1998). Most attention has been focused on the influence of UV radiation on the phenylpropanoid pathway, as this is involved in color, tannin formation, and the production of antioxidants. One of the most interesting aspects of UV regulation is that the response takes place after veraison when the berries soften, irrespective of UV exposure levels (Jordan personal observation and see above relating to UV-B and developmental responses). This regulation has been linked to the levels of carbohydrate within the fruit

(Lenk et al., 2007). Thus, as the sugar accumulates within the berry, a point is reached and thereafter biosynthesis of flavonoid compounds takes place. This is particularly important when the formation of another chemical, resveratrol (trans-3,5,4'-trihydroxystilbene), is considered. Resveratrol is thought to play an important role in plant defense and to have a beneficial role in human health (Pan et al., 2009). The pivotal enzyme in the biosynthesis of resveratrol is stilbene synthase, STS (Pan et al., 2009). This enzyme and the subsequent synthesis of resveratrol takes place mostly pre-veraison in contrast to the biosynthesis of the pathway to anthocyanins, flavonols, etc., through the first committed enzyme step, CHS (chalcone synthase). Both CHS and STS use coumaroyl CoA as a substrate. Although more understanding is needed, it is likely that this split in the pathway to either anthocyanin or resveratrol is a key to the outcome of significant grape chemistry. Most importantly, UV radiation has a strong influence on both of these pathways although at different stages of development (pre- and post-veraison). Another important area of vine biochemistry is the metabolism of amino acids. The assimilation of amino acids takes place primarily in leaves and this assimilate is then redistributed from the leaves to the grape berries. Amino acids are very important for viticulture and oenology as they are precursors for the phenolic compounds, aromatics (such as methoxypyrazine and thiols), and are necessary for fermentation (YAN: yeast assimilable nitrogen). However, very little is known about their UV regulation. Schultz et al., (1998) showed that over two seasons, the total amino acid levels were higher when UV-B was reduced to approximately (10% of ambient). Furthermore, there was a qualitative change in amounts of amino acids that contribute substantially to YAN, such as arginine and glutamine, which increased in lower UV-B conditions. A non-YAN amino acid, proline, also increased under lower UV-B. Proline is an amino acid that frequently shows a response to stress, so it is no surprise that it responded to UV-B. In contrast to these results, Keller and Torres-Martinez (2004) found no change in amino acid composition under UV-B treatments, although they did find differences in polyphenolics. The differences in response could be due to significantly different experimental treatments (vineyard screening versus potted vines). In UV screening vineyard trials at Lincoln University, New Zealand, both qualitative and quantitative changes to amino acids and flavonoids were detected (Jason et al. personal communication 2009). Over the 2008 and 2009 seasons, UV screens have been placed over rows of Sauvignon blanc grapes in the Lincoln vineyard to study the effects of UV-B and UV-A radiation in "leaf plucked" vines and in comparison to control vines (no screens and no leaf removal). UV-exposure clearly has a dramatic effect on the physical appearance of the berries with specific pigmentation relating to the extent of exposure. The increase in pigmentation appeared to increase post-veraison and would support previous findings. Significantly, the levels of certain amino acids in the berries relate to the presence of leaves remaining over the fruiting zone. Wines have been made from these treatments and show differences in appearance and taste from the initial pressings to the final product. It is very clear from these studies (and in commercial vineyard observations) that there is a significant role of UV-B in the development of grapes.

## 22.6 CONCLUDING COMMENTS

Substantial progress has been made toward understanding the molecular mechanisms involved in UV-B responses since the review of Mackerness and Jordan (1999). Most importantly, it is recognized that UV-B is part of the natural light environment and can influence photomorphogenetic responses in addition to acting as an abiotic stress. This has led to the realization that the UV-B response may well be induced at different wavelengths and under different fluence levels. Progress has been made to elucidate the specific photoreceptor(s) involved in UV-B responses and the signal transduction pathways downstream from perception. Further progress is however, needed to confirm and characterize potential UV-B photoreceptors. Microarray technology has opened up a genome-wide spectrum of UV-B responses and pointed to interesting areas for further study, such as UV-B regulation of chromatin remodeling. Finally, from studies on grape berries, it is apparent that UV-B radiation can have an impact on the quality of important commercial products, such as wine.

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# 23 Effect of High Temperature and UV-A Radiation on Photosystem II

*E.L. Apostolova and A.G. Dobrikova*

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## 23.1 INTRODUCTION

Photosynthetic organisms are directly exposed to changes in the environment and their survival depends on their ability to acclimate to such changes. The photosynthetic membranes are very sensitive to stress factors such as temperature above and below normal physiological range and UV radiation. The most detrimental component of the natural sunlight is UV-A (320–400 nm), which passes through the stratospheric ozone layer almost unattenuated and damages plant photosynthesis [1–5]. The changes in photosynthetic membranes depend on growth conditions and plant species. The primary target of stress factors action in plants and algae is photosystem II (PSII), since one of the most sensitive site is the oxygen-evolving complex (OEC) [2,3,5]. The influence of high temperature and UV-A radiation on PSII, as well as the role of the organization of LHCII-PSII (light-harvesting antenna and core complex of PSII) supercomplex on the damaging effect of these stress factors are reviewed here.

## 23.2 STRUCTURE AND FUNCTION OF LHCII-PSII SUPERCOMPLEX

Photosystem II is a multisubunit chlorophyll–protein complex, embedded in the thylakoid membrane of chloroplasts, which drives electron transfer from water to plastoquinone to produce molecular oxygen and protons using energy derived from light [6,7]. In green plants, the PSII core complex is surrounded by the light-harvesting complex (LHCII) and thus it is called the LHCII-PSII supercomplex, which consists of more than 30 proteins [7,8]. The reaction center is composed of a D1/D2 heterodimer and a few intrinsic low-molecular-weight polypeptides. The reaction center is surrounded by the core antenna chlorophyll (Chl)-*a* binding proteins, CP43, and CP47. The LHCII-PSII supercomplex contains three extrinsic proteins of 33, 23, and 17 kDa associated with the luminal surface of PSII reaction center complex, forming OEC [8,9].

The water oxidation on the PSII donor side is catalyzed by a cluster of four Mn ions of OEC [6,8,10], and the liberated electrons are transferred to the reaction center chlorophyll,  $P_{680}$ , via the immediate redox-active tyrosine residue,  $Y_Z$  (Tyr-161), located on the D1 protein. PSII contains accessory redox-active tyrosine,  $Y_D$  (Tyr-160) on the D2 subunit, which is not connected to the water-oxidizing complex. The functional conformation of the Mn cluster is expected to be maintained by a 33 kDa hydrophilic protein subunit of OEC attached to the luminal side of the D1/D2 heterodimer. During the oxidation of two water molecules to one oxygen molecule and protons, the OEC cycles through five intermediate redox states termed  $S_0$  to  $S_4$ . The most reduced state is  $S_0$ , while  $S_1$ ,  $S_2$ , and  $S_3$  represent higher oxidation states and the  $O_2$  being evolved at the transient of  $S_4$  to  $S_0$  state [11–13]. In darkness, the  $S_0$  and  $S_1$  states are stable, while  $S_2$  and  $S_3$  revert to  $S_1$  in a few minutes. It is suggested that there are two different mechanisms for oxygen production [14,15]: the cooperative mechanism is realized by the diffusion of oxygen precursors within the different OECs (mainly PSII $\beta$  centers) and is characterized by a rate constant lower than that of the noncooperative Kok's mechanism realized by PSII $\alpha$  centers [15].

This linear electron transfer reaction in PSII catalyzes the light-induced water–plastoquinone oxidation–reduction with a high quantum efficiency. In addition to the main linear electron transfer, it has been proposed that there is a low quantum yield cyclic electron transfer around PSII, which mediates electron flow from  $Q_B$  to  $P_{680}$  [16]. The cyclic electron transfer may protect PSII against photoinhibition by preventing over-reduction of  $Q_A$  and  $Q_B$  on the acceptor side and accumulation of long-lived  $P_{680}^+$  on the donor side [17]. On the acceptor side of PSII, the electron produced by the light-induced charge separation event reduces a pheophytin molecule and then the first,  $Q_A$ , and the second,  $Q_B$ , plastoquinone (PQ) electron acceptor [6].

Outer antenna of PSII units (LHCII complex) composed of proteins that bind Chl *a* and *b*, is primarily composed of major antenna proteins (Lhcb1–3), as well as less abundant minor antenna proteins (Lhcb4–6) [7,18]. The major LHCII is believed to be organized in hetero-trimeric units (or oligomers) in thylakoid membranes and to bind 50% of the chlorophyll molecules present in chloroplasts [18]. There are few models for the LHCII proteins arrangement in PSII units, which show difference in the stoichiometries of the proteins [7,18]. The role of LHCII has been recognized in stabilizing the membrane structure and in particular in the stacking of adjacent granum membranes [19,20].

LHCII plays an important role in the regulation of light absorption and energy transfer to the reaction centers, therefore any damage of this complex would result in multiple effects on the photosynthetic apparatus. The relative light-harvesting capability of the two photosystems (PSI and PSII) can be regulated by redox-regulated reversible phosphorylation of LHCII proteins—a process known as state transitions [21]. This process correlates with the redistribution of peripheral antenna proteins and, thus, the excitation energy between the two photosystems. The phosphorylation/dephosphorylation of LHCII has been shown to be specifically influenced by environmental factors, mainly light and temperature, and is implicated in various adaptive and regulative responses of the photosynthetic apparatus [22–24]. During this process, part of LHCII (after phosphorylation) is transferred from the stacked to the unstacked region of the thylakoid membranes. The phosphorylation of the LHCII

proteins considerably facilitates the heat- and light-induced reorganizations of thylakoid membranes and thus enhances the structural flexibility of the membrane architecture [25].

### 23.3 PHOTOSYNTHETIC RESPONSE TO HIGH TEMPERATURE

Assessment of heat tolerance is of primary importance in programs designed to improve heat stability in crop plant. High temperature stress is one of the most prominent abiotic stresses affecting plants. Exposure of plants to heat stress can be accompanied by other stresses. The plants from different geographic zones could be adapted to the cool or hot environment. The extent of the damage of the photosynthetic apparatus caused by exposure to high temperature differs depending on the plant species, the state of growth, the temperature, the rate of temperature change as well as the duration of temperature treatment [26–30].

The most sensitive component to high temperature in photosynthetic membranes is LHCII-PSII supramolecular complex [31]. PSI is also destroyed by heat treatment though it exhibits higher stability than PSII [32]. Temperature deactivation of PSII includes functional separation of LHCII from PSII core complex [28,33–36] and damage of donor [37,38] and acceptor part of PSII [39–41]. Havaux and Strasser [42] showed that the photosynthetic oxygen evolution was irreversibly inhibited after short exposure to the heat (42°C) of pea leaves. In addition, Shi et al. [43] reported that the inactivation of the oxygen evolution is not associated with any major protein secondary structural changes. Havaux [44] suggested that temperatures up to 42°C have no inhibitory effect on the acceptor side of PSII. This author showed that the electron transfer from primary ( $Q_A$ ) to the secondary ( $Q_B$ ) electron acceptor of PSII and the fraction of  $Q_B$ -non-reducing PSII centers remain unchanged. A large decrease in the electron flow from  $Q_A$  to  $Q_B$  became prominent after heating at around 50°C [41]. Zhang and Sharkey [45] proposed that the heat alters the redox balance away from PSII toward PSI and that the regulation of the cyclic electron transport around PSI protects the photosynthetic apparatus from heat-induced damage.

#### 23.3.1 STRUCTURAL CHANGES IN PHOTOSYNTHETIC APPARATUS UNDER HIGH TEMPERATURE

Vani et al. [35] showed that *in vivo* exposure of rice seedling to elevated temperature (40°C) did not cause any changes in the protein or pigment content of thylakoid membranes, but caused disorganization of membrane structure and of some thylakoid membrane complexes, which resulted in a loss of their photochemical functions. Exposure of chloroplasts (*in vitro*) to heat treatment with temperature higher than 35°C is known to lead to changes in the organization of thylakoid membranes [27,28,46]. Incubation at 35°C–45°C causes complete destacking of thylakoids accompanied with disruption of chlorophyll–protein complexes of the both photosystems and vesiculation of thylakoid membranes [27,46]. At temperatures around 50°C and above packing of thylakoids in grana is changed and formed strands of pseudograna with increased membrane stacking, since the membrane–membrane interactions in these pseudogranas are of another origin than in the normal grana [46].

It is well known that LHCII takes part in temperature-induced structural changes [36,47,48]. The changes in the structural organization of thylakoid membranes above 40°C are accompanied by dissociation of LHCII from PSII core complex and its diffusion from the granal to the stromal region [34,49]. Pastenes and Horton [50] have shown that high temperatures, which lead to reversible inhibition of photosynthesis, influence on the redistribution of the excitation energy between the two photosystems and the ratio between two subpopulation of PSII: PSII $\alpha$  and PSII $\beta$ . Some of these investigations are made *in vitro*, but altered energy distribution between PSII and PSI was also shown *in vivo* after treatment of leaves [51]. Recent results [36] have shown that the temperature treatment *in vivo* (around 42°C) influences the pigment–protein and pigment–pigment interaction in LHCII, and leads to the increase of the migration of phosphorylated LHCII from stacked to unstacked thylakoid membranes.

Temperature-induced structural rearrangement in isolated thylakoid membranes was also revealed by differential scanning calorimetry and circular dichroism spectroscopy [52]. It was suggested that several thermo-induced structural transitions occurred: the first begins with the unstacking of the membranes followed closely by a lateral disassembly of the LHCII-containing macrodomains in the granum, after which the trimers of LHCII are transformed into monomers during heating around 60°C [52]. The temperature of the trimer-to-monomer transition for isolated LHCII trimers was found to be 55°C [53].

### **23.3.2 INFLUENCE OF THE LIPID COMPOSITION ON THE THERMOSTABILITY OF LHCII-PSII SUPERCOMPLEX**

Threshold temperatures for structural and functional changes in thylakoid membranes strongly depend on growth temperature of photosynthetic organisms. Many authors suggested that lipid composition has important role for temperature stability of the thylakoid membranes [54–57]. Aminaka et al. [58] suggested that one of the major factors that change the thermostability of PSII, after acclimation to high temperature, is the change in the fluidity of the membranes. The temperature-induced changes in membrane fluidity are one of the immediate consequences in plants during temperature stress. Mutants deficient in fatty acid desaturation showed strong tolerance to high temperature [56,57,59,60]. On the other hand, the changes in lipid composition have influence on the membrane protein organization and function [61–64]. Structural coupling between major proteins and lipids during the formation of photosynthetic apparatus and lipid–protein interactions are required for the stability and protection of thylakoid membrane protein assemblies [26].

Temperature denaturation of PSII accompanied with big changes in the lipid bilayer of thylakoid membranes has been reported by several studies [27,28,31]. Increase in temperature leads to increased molecular movement (fluidity) of the membrane lipids [54] and subsequent formation of non-bilayer lipid structure [27]. It has been shown that at temperatures above 45°C–55°C, phase-separation of the non-bilayer-forming lipids from the bulk phase occurs, which results in breakdown and vesiculation of the thylakoids [27,28]. The main consequences of these lipid changes are destabilization of lipid–protein interactions and the alteration of the structure and function of PSII. It has been also proposed that non-bilayer-forming lipids are involved in the packing of the LHCII supramolecular complex, which correlates with the reported conversion of the major LHCII from trimeric to monomeric forms during heat stress [47].

### **23.3.3 EFFECT OF HIGH TEMPERATURES ON THE OXYGEN-EVOLVING COMPLEX**

The most sensitive component of photosynthetic apparatus to heat stress is the OEC. Nishiyama et al. [65] suggested that OEC determined the heat sensitivity of isolated thylakoid membranes. The changes in OEC are connected with the release of manganese atoms, which catalyze the O<sub>2</sub> evolution, from the PSII core causing a loss of the oxygen-evolving activity [37]. Enami et al. [38] suggested that the release of the Mn-stabilizing 33 kDa extrinsic protein occurs first followed by the liberation of manganese atoms and inhibition of oxygen evolution. It was supposed that 33 kDa proteins of OEC released from PSII core complex during heat treatment with temperature up to 40°C become loosely bound and rebind to the complex when the chloroplast membranes are cooled down to 25°C [41]. At temperatures higher than 50°C, the 33 kDa protein is denaturated and the changes are irreversible.

It is well known that chloride ions are very important for the normal function of PSII and their loss causes inactivation of OEC [66]. Chloride ions not only take part in the process of oxygen evolution but they also preserve PSII from temperature-induced damage [67]. It is suggested that there are two sites of chloride binding: high-affinity and low-affinity binding sites [68,69]. The high-affinity (tightly bound, slow exchanged) site of chloride is affected earlier (around 37°C) while low-affinity (loosely bound, fast exchanged) site is affected at higher temperature (42°C) for thylakoid membranes [70].

### 23.3.4 DEGRADATION OF THE COMPONENTS OF PSII UNDER HIGH TEMPERATURE

Vani et al. [35] suggested that the loss in quantum yield accompanied by decrease in number of PSII active reaction centers, could be due to possible uncoupling of light-harvesting antenna from reaction center brought by heat treatment and/or the reduction of core antenna proteins, CP43 and CP47, which play important role in excitation energy transfer from the peripheral antenna, LHCII, in higher plants to PSII reaction center. Furthermore, they also play an important role in maintaining the structural integrity and oxygen-evolving capacity of PSII [7]. High-temperature treatment of thylakoid membranes in dark leads also to the degradation of D1 protein, as maximal degradation is occurring at 45°C concomitant with the release of 23 kDa fragment [71].

Ristic et al. [72] suggested that heat-induced damage of thylakoid membranes and chlorophyll loss are closely associated in winter wheat. The release by heat of two out of four Mn atoms from OEC results in the complete inactivation of OEC activity without any significant loss of proteins [37].

Differential scanning calorimetry (DSC) was employed by several laboratories to study thermostability of thylakoid membranes and isolated membrane complexes [52,73–75]. It has been shown that thylakoid membranes from different higher plants have six or seven major endotherms in the 40°C–100°C range that can be attributed to protein denaturation as DSC peaks depend on plant species, pH and ion strength of the suspending buffer. It has been suggested that the denaturation temperature of the PSII core complex is in the range 51°C–60°C [74]. Smith et al. [73] have suggested that the main DSC transition at about 76°C in thylakoid membranes from spinach derives from LHCII. Our investigation of the mutant thylakoid membranes with different amount and degree of LHCII oligomerization show that a decrease in the amount of LHCII as well as the oligomerization of the complex lead to strong decrease of the calorimetric enthalpy of the transition at 76°C (unpublished results). In addition to this, our results from DSC measurements showed that the thermodynamic parameters of other transitions, which correlate with proteins of PSII supercomplex, are also influenced by the modification of LHCII (unpublished results).

## 23.4 PHOTOSYNTHETIC RESPONSE TO UV-A RADIATION

The information related to the low-energetic, but more intense, UV-A (320–400 nm) radiation on plants is limited compared with visible and UV-B effects. It is shown that the primary target of UV-A radiation in thylakoid membranes is the PSII complex [2,3]. The UV-A-induced changes in PSII of higher plant leaves were found to be synergistically accelerated by high growth temperature suggesting that the radiation poses a considerably higher risk for plants in tropical regions [76]. Studies performed in intact and isolated systems indicated that UV-A induces oxidative damage and inhibition of growth [77]. It has already been shown that UV-A radiation has effect on the induction of the lipid peroxidation of biological membranes, polyunsaturated fatty acids, and phospholipid liposomes [78]. Moorthy and Kathiresan [79] suggested that UV-B tolerance of plants correlates with the degree of the unsaturation of fatty acids. UV-A radiation has been found to decrease the ratio of unsaturated to saturated fatty acids in thylakoid membranes from cotton, which could be a result from lipid peroxidation, and leads to changes in membrane fluidity [80].

Simultaneous monitoring of the damage induced by both UV-A and UV-B has shown that the damage of the photosynthetic apparatus in seedlings (leaves) is less as compared to that in a UV-B-treated one [81], as UV-A radiation considerably restores the injuries of PSII caused by UV-B irradiation.

It was also suggested that UV-A stress not only depended on light intensity but also on the exposure time, as an increase in the UV-A exposition time significantly caused stress in photosynthesis and a decrease in the protein content [82,83].

### 23.4.1 STRUCTURAL CHANGES IN THE PHOTOSYNTHETIC APPARATUS UNDER UV-A IRRADIATION

Renger et al. [84] have shown that similarly to the temperature treatment, UV radiation leads to functional disconnection of the LHCII from the core complex of PSII in isolated thylakoid membranes. Their structural integrity is altered, from slight swelling and dilation of membranes to physical disruption of the chloroplasts, in plants exposed to UV-B radiation for various periods of time [85]. On the other hand, the irradiation of isolated pea thylakoid membranes with UV-A was revealed to lead to an increase in the energy transfer from PSII to PSI, which could be a result of the disconnection of LHCII from PSII [83]. It has been shown that UV-A irradiation influences the energy transfer in LHCII-PSII supercomplex too [83].

### 23.4.2 EFFECT OF UV-A RADIATION ON THE LHCII-PSII SUPERCOMPLEX

UV-A irradiation is shown to damage the primary photochemistry of PSII to larger extent than PSI [2,76], but the underlying injured mechanisms are not understood in detail. The damaging mechanism of UV-A is very similar to that induced by UV-B radiation [3]. Several UV-sensitive sites were supposed to exist in PSII complex, including the redox-active tyrosine, the Mn cluster on the donor side, and the plastosemiquinones on the acceptor side [2,3,5,86]. The harmful effects of UV-A radiation on PSII function were manifested as inhibition of electron transport, photosynthetic oxygen evolution, modified  $Q_B$  binding affinity, as well as a direct damage on key components such as D1 and D2 proteins of PSII [2,3,86].

The OEC has been suggested to be the most sensitive target [3]. The primary site of UV-A radiation damage is thought to be the catalytic manganese cluster of the OEC [3]. The inactivation of the electron transport between the Mn cluster and the tyrosine electron donors and/or damage of the protein environment around the Mn cluster by reactive free radical species were proposed to be the immediate cause for the loss of  $O_2$  evolution by UV radiation [3]. One possible reason for the UV-induced inhibition of oxygen evolution could be the absorption of UV light by Mn ions in oxidation states, Mn (III) and Mn (IV), which constitute the catalytic site of the OEC in their higher oxidation states [3,5]. Some observations suggested that the  $S_1 \rightarrow S_2$  and  $S_2 \rightarrow S_3$  redox transitions of the Mn cluster are accompanied by absorption changes in the UV region [3,5]. The UV-A-induced changes in the OEC influence the  $S_0$ - $S_1$  state distribution in the dark, i.e., the  $S_0$  populations of PSII centers increase in darkness [3,83]. It can be proposed that higher amount of Mn ions in Mn (III) oxidation state (i.e., increased number of PSII centers in  $S_1$  state) in some plant species would be one of the possible reasons for the higher sensitivity of their photosynthetic apparatus to UV radiation.

The sensitivity of redox-active tyrosine to UV-B radiation is determined by their absorption in the UV range [5]. The oxidized radical form of tyrosines absorbed around 300 nm, which extended into UV-A region. Vass et al. [3] observed that both redox-active tyrosines— $Y_Z$  on the D1 protein and  $Y_D$  (on the D2 protein) and its environment—are damaged by UV-A.

It is supposed that  $Q_A$  and  $Q_B$  acceptor molecules are also potential targets of UV radiation, which leads to their destruction. Plastosemiquinones have an absorption band at around 360–380 nm; therefore, they may be potential UV-A targets [3]. On the other hand, injury of D1 and D2 protein subunits from the reaction center of PSII during UV-A irradiation [76,87,88] leads to the modification of the surroundings of  $Q_A$  and  $Q_B$  [2,3,76], which decreases the binding affinity of PQ at the  $Q_B$  site. It was found that the UV-A damage has a more pronounced effect on D1 than on D2 [2]. The UV-A-induced breakdown product of D1 is a 20 kDa fragment, which has the same size as breakdown products induced by UV-B radiation [3].

The changes in PSII complex are accompanied with an inhibition of the PSII-mediated electron transport and inhibition of the photosynthetic oxygen evolution due to the inactivation predominantly of functionally active PSII $\alpha$  centers in the grana domains, which are characterized with a large antenna size [83,89].



### 23.4.3 EFFECT OF UV-A RADIATION ON THE PHOTOSYNTHETIC PIGMENTS

The changes in structure and function of UV-irradiated plants are accompanied with decrease in pigment content [4,5]. Photosynthetic pigments show different sensitivity to UV radiation. The influence on the carotenoids is smaller than that on chlorophylls [90]. It has been shown that UV-B has different effect on Chl *a* and Chl *b*. The decrease of Chl *b* is bigger than that of Chl *a*, which could be a result of its degradation and/or changes in its biosynthesis [91]. The effect of UV-B radiation on the ratio Chl *a/b* depends on the plant species and growth conditions [4,5].

The irradiation of macroalgal species with UV radiation (UV-A and UV-B) causes loss of Chl *a* [92]. The decrease of Chl *a* is accompanied with loss of proteins only in deepwater species. It has been shown that treatment with high UV-B irradiance causes decrease in chlorophylls of light-harvesting complexes and degradation of LHCII [79]. The investigation of the effect of UV-A on wheat showed that the short irradiation has little effect on the level of photosynthetic pigments and Chl *a/b* ratio [76]. Recently, it has been shown that long exposure of UV-A (more than 24 h) leads to Chl *a* degradation in lichens [82].

## 23.5 ROLE OF THE ORGANIZATION OF LHCII-PSII SUPERCOMPLEX FOR THE SENSITIVITY OF THE PHOTOSYNTHETIC APPARATUS TO HIGH TEMPERATURE AND UV-A RADIATION

The different sensitivity of plant species to high temperature and UV radiation puts a question for the role of the organization of the photosynthetic apparatus to environmental stress response. Developing thylakoid membranes during greening, mutants with different amount and organization of LHCII, as well as the manipulation of LHCII size with exogenous polyamines are used by several authors as model systems for studying the role of the antenna size of PSII in stress stability [26,30,83,93].

### 23.5.1 SENSITIVITY OF THE PHOTOSYNTHETIC APPARATUS IN GREENING PLANTS TO HIGH TEMPERATURE

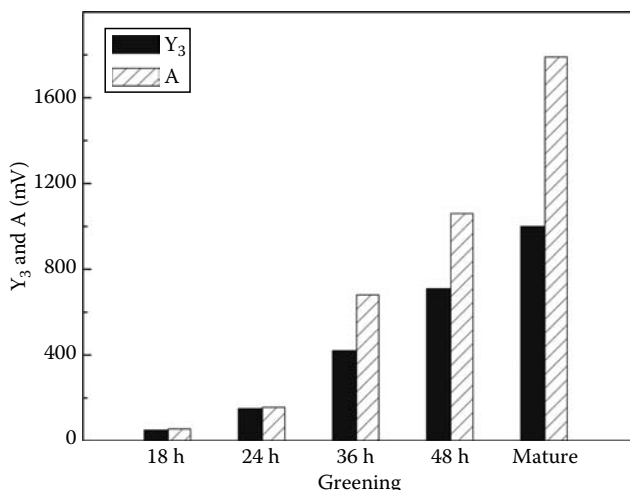
The development of the thylakoid membrane of greening plant seedlings is connected with the monotonic accumulation of chlorophylls and light-harvesting chlorophyll *a/b*-binding polypeptides (LHCII) associated with PSII apoproteins [26,94,95]. The content of 18:3 fatty acids increases during greening and facilitates the packing of larger protein assemblies in the thylakoid membranes [26]. LHCII is known to be in the monomeric form in the early phases of greening [95,96] and associates into trimers or larger aggregates during development [26]. It has been supposed that formation of higher-order oligomeric LHCII complexes is related to grana stacking formation in chloroplasts. Some authors reported an increase in the number of thylakoids per granum during the greening period [96,97], which correlated with an increase in the intensity of the 77 K fluorescence emission at 735 nm, associated with PSI [94]. The appearance of redox-controlled LHCII phosphorylation induced by light was detected only after 24–36 h of greening, which is suggested to correlate with the segregation of the thylakoid protein components and the development of grana stacks consisting of at least four thylakoids per stack [96,97].

Previous data showed that the low-temperature chlorophyll fluorescence emission ratio of PSI (735 nm) to PSII (685 nm), corresponding to energy distribution between the two photosystems, strongly depends on the ratio of oligomeric (hetero-trimeric) to monomeric LHCII in thylakoid membranes [13]. Our results showed that the fluorescence emission ratio  $F_{\text{PSI}}/F_{\text{PSII}}$  for developing thylakoid membranes increased after 24 h illumination of barley leaves (data not shown), which probably correlates with the appearance of the granal domains and trimeric structure of LHCII.

Investigations of developing thylakoid membranes using Fourier transform infrared and spin label electron paramagnetic resonance spectroscopic techniques suggested that the greening is

accompanied with the reorganization of the membrane protein assemblies and alteration on the protein–lipid interactions [26]. These changes in the organization of the membrane complexes influence the photosynthetic oxygen evolution (flash-induced oxygen yields and initial oxygen burst under continuous illumination). The amplitudes of the oxygen burst under continuous illumination ( $A$ ) and the oxygen yields after third flash ( $Y_3$ ) are usually used for the characterization of the photosynthetic oxygen evolution [13,83]. Studies of the oxygen production of developing thylakoid membranes from greening barley leaves using oxygen rate electrode have shown gradual increase of flash-induced oxygen yields ( $Y_3$ ) and initial oxygen bursts amplitudes ( $A$ ) under continuous illumination during greening (Figure 23.1). The oxygen production can be registered only after an 18 h greening period (Figure 23.1). The amplitude of the oxygen burst, which is connected with the amount of the functionally active PSII centers [13], rises during greening, stronger than the flash-induced oxygen yields. The oxygen induction curves under continuous illumination of developing thylakoid membranes after 18 h and 24 h of greening are characterized with the smoothing of the initial burst (data not shown) and lower values of the amplitudes of oxygen burst (Figure 23.1). These observations reveal that in earlier development of thylakoid membranes, the oxygen evolution is realized by the cooperative mechanism from PSII centers in stroma lamellae, PSII $\beta$  [13]. On the other hand, the increase of flash-induced oxygen yields ( $Y_3$ ) as well as the appearance of the characteristic oscillations over the 36 h of greening is accompanied by an increase of the functionally active PSII centers evolving oxygen by noncooperative mechanism realized by PSII $\alpha$  centers in grana domains. Similar characteristics of the photosynthetic oxygen production to those after 24 h period of greening were observed for *Chlorina f2* barley mutant, which has strongly reduced the antenna size of PSII and lack of oligomeric structure of LHCII [13].

Previous data suggested that the increase of the oxygen flash yields and the initial burst amplitudes (sharpness of the peak) corresponds to higher ratio of LHCII per unit PSII reaction center and enhanced amount of PSII $\alpha$  in comparison to PSII $\beta$  functionally active centers [13]. Therefore, the oxygen parameters for characteristics of thylakoid membranes during greening indirectly reveal the accumulation of the oligomeric forms of LHCII (or thylakoid stacking formation) over the 36–48 h period of illumination of dark-grown barley.



**FIGURE 23.1** Photosynthetic oxygen evolution of thylakoid membranes isolated from greening barley seedlings for different times of illumination ( $130 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) (unpublished data).  $Y_3$ —flash-induced oxygen yield after the third flash;  $A$ —amplitude of initial oxygen burst under continuous irradiation with white light ( $450 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). For details of measurements and calculations of the parameters of the oxygen evolution, see Apostolova et al. [13].

**TABLE 23.1**  
**Effect of High-Temperature Stress (5 min) on the Oxygen Evolution**  
**Parameters (% of the Control) of Isolated Thylakoid Membranes from**  
**Barley Wild Type, *Chlorina f2* Mutant and Greening Barley Seedlings**  
**for Different Time of Illumination (130  $\mu\text{mol Photons m}^{-2} \text{s}^{-1}$ )**

| <i>T</i> (°C) | Greening Plants       |          |                       |          |                       |          |                       |          | Wild Type             |          | Chlorina<br><i>f2</i> |          |
|---------------|-----------------------|----------|-----------------------|----------|-----------------------|----------|-----------------------|----------|-----------------------|----------|-----------------------|----------|
|               | 18h                   |          | 24h                   |          | 36h                   |          | 48h                   |          | <i>Y</i> <sub>3</sub> | <i>A</i> | <i>Y</i> <sub>3</sub> | <i>A</i> |
|               | <i>Y</i> <sub>3</sub> | <i>A</i> | <i>Y</i> <sub>3</sub> | <i>A</i> | <i>Y</i> <sub>3</sub> | <i>A</i> | <i>Y</i> <sub>3</sub> | <i>A</i> |                       |          |                       |          |
| 25            | 100                   | 100      | 100                   | 100      | 100                   | 100      | 100                   | 100      | 100                   | 100      | 100                   | 100      |
| 30            | —                     | —        | 43                    | 71       | 72                    | 82       | 80                    | 90       | 85                    | 90       | 74                    | 87       |
| 35            |                       |          | 29                    | 36       | 43                    | 52       | 47                    | 73       | 63                    | 74       | 59                    | 60       |
| 40            |                       |          | —                     | —        | 18                    | 29       | 28                    | 40       | 34                    | 54       | —                     | —        |
| 45            |                       |          |                       |          | —                     | 8        | 10                    | 3        | 11                    | 18       |                       |          |

*Notes:* Unpublished data; *Y*<sub>3</sub>, flash-induced oxygen yield after the third flash; *A*, amplitude of initial oxygen burst under continuous irradiation with white light (450  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). For details of measurements and calculations of the parameters of the oxygen evolution see Apostolova et al. [13].

The thermal stability of the photosynthetic oxygen evolution of developing thylakoid membranes increases during greening (Table 23.1). The data show that thylakoid membranes from 24 h greening barley leaves have similar thermal sensitivity as *Chlorina f2* mutant thylakoids, but smaller than that of 36 h and 48 h greening plants. The mature plants (barley wild type) possess the highest stability. In both cases (24 h of greening and *Chlorina f2*), the full inhibition of the oxygen-evolution parameters is registered after heat stress at 40°C. These data are in agreement with observations for the thermostability of PSII in leaves of *Chlorina f2*, which reveal that the donor side of PSII appeared to be less stable in *Chlorina f2* than in wild type [30]. These results support the idea that larger amount of LHCII stabilizes the function of OEC during high-temperature treatment.

**23.5.2 ROLE OF LHCII SIZE FOR THE SENSITIVITY OF THE PHOTOSYNTHETIC APPARATUS TO UV-A RADIATION AND HIGH-TEMPERATURE TREATMENT**

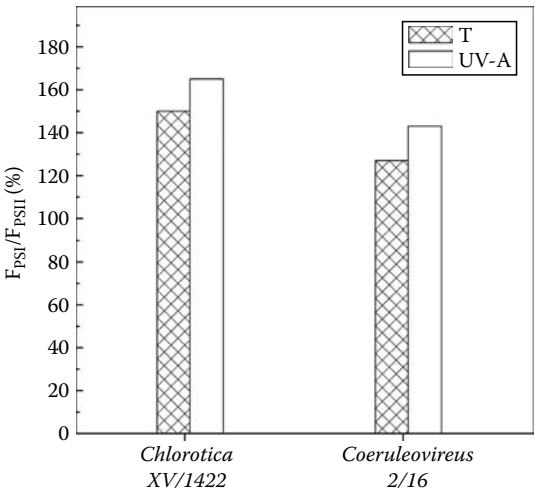
Recent investigations on pea mutants suggested a relationship between the amount and organization of LHCII and the tolerance of the photosynthetic apparatus to UV-A radiation [83] and high temperature (unpublished data). Here, we compare the effects of the high temperature and UV-A radiation on the two pea mutant forms: *Chlorotica XV/1422* and *Coeruleovireus 2/16* (Table 23.2 and Figure 23.2). The ratio of the oligomeric (LHCII<sub>o</sub>) to monomeric (LHCII<sub>m</sub>) forms of LHCII, as well as the ratio of LHCII to PSII core complex (LHCII/PSII) are smaller in *Chlorotica XV/1422* (LHCII<sub>o</sub>/LHCII<sub>m</sub> = 2.45 and LHCII/PSII = 1.97) in comparison to the mutant *Coeruleovireus 2/16* (LHCII<sub>o</sub>/LHCII<sub>m</sub> = 6.62 and LHCII/PSII = 3.21) [13,98,99]. The influence of high temperature and UV-A radiation on the energy distribution between the two photosystems and on the photosynthetic oxygen evolution (flash-induced oxygen yields, amplitudes of oxygen burst under continuous irradiation, and PSII-mediated electron transport) of isolated thylakoid membranes is shown in Figure 23.2 and Table 23.2. The 77 K fluorescence emission ratio  $F_{\text{PSI}}/F_{\text{PSII}}$  (taken as index for the extent of energy spillover from PSII to PSI) increases under UV-A and high-temperature treatment (Figure 23.2) as a result from the separation of LHCII from PSII core complex and its migration to the stroma thylakoid membranes during the action of these stress factors [34,49,84]. The higher degree of increase of the  $F_{\text{PSI}}/F_{\text{PSII}}$  ratio for the *Chlorotica XV/1422* compared to the *Coeruleovireus 2/16*

**TABLE 23.2**  
**The Photosynthetic Oxygen Evolution of Thylakoid Membranes from Pea Mutants: *Chlorotica* XV/1422 and *Coeruleovireus* 2/16**

| Plant Type                 | Oxygen Evolution (% of the Control) |                |     |
|----------------------------|-------------------------------------|----------------|-----|
|                            | H <sub>2</sub> O → BQ               | Y <sub>3</sub> | A   |
| <i>Chlorotica</i> XV/1422  | 100                                 | 100            | 100 |
| T (°C) <sup>a</sup>        | 51                                  | 34             | 31  |
| UV-A <sup>b</sup>          | 29                                  | 9              | 15  |
| <i>Coeruleovireus</i> 2/16 | 100                                 | 100            | 100 |
| T (°C) <sup>a</sup>        | 85                                  | 63             | 76  |
| UV-A <sup>b</sup>          | 42                                  | 20             | 44  |

*Notes:* Effect of high-temperature stress (5 min) and UV-A radiation (2h) on the values of PSII-mediated electron transport (H<sub>2</sub>O → BQ) and parameters of the oxygen evolution: Y<sub>3</sub>—flash-induced oxygen yield after the third flash and A—amplitude of initial oxygen burst under continuous irradiation with white light (450 μmol photons m<sup>-2</sup> s<sup>-1</sup>) (for details see Ref. [83]). The presented values for electron transfer H<sub>2</sub>O → BQ are measured at 45°C, while these for Y<sub>3</sub> and A—at 40°C, because the oxygen evolution without artificial electron acceptor is fully inhibited at 45°C in mutant *Chlorotica* XV/1422.

<sup>a</sup> Based on unpublished data.  
<sup>b</sup> Adapted from Ivanova, P.I. et al., *Radiat. Environ. Biophys.*, 47, 169, 2008.



**FIGURE 23.2** Effect of temperature treatment (50°C, 5 min) and UV-A radiation (3 h) on the 77 K chlorophyll fluorescence emission ratios (for excitation at 436 nm) of thylakoid membranes from two pea mutants (*Chlorotica* XV/1422 and *Coeruleovireus* 2/16). Data are presented as percentage of nontreated control thylakoid membranes. (Data for UV-A radiation are modified from Ivanova, P.I. et al., *Radiat. Environ. Biophys.*, 47, 169, 2008.)

suggests that the influence of UV-A and temperature on the rearrangement of pigment–protein complexes in the thylakoid membranes with smaller antenna size is stronger than that in the membranes with bigger antenna size.

The changes in the membrane organization under stress treatments influence the oxygen evolution as the degree of inhibition depends on the organization of LHCII-PSII supercomplex. Comparison of the sensitivity of the oxygen evolution after high-temperature and UV-A radiation treatment in the two mutant forms shows that the inhibition is bigger in *Chlorotica XV/1422* in comparison to *Coeruleovireus 2/16* (Table 23.2), i.e., the damage effect of these stress factors on the oxygen-evolution parameters decreases with the increase in the amount and the oligomerization of LHCII. In addition, it has been supposed that the inhibition of the photosynthetic oxygen evolution under UV-A radiation is due predominantly to the inactivation of functionally active PSII $\alpha$  centers in grana domain, which are characterized with larger antenna size than PSII $\beta$  [83].

## 23.6 CONCLUSIONS

Many studies have shown that the donor side of PSII (OEC) is the most sensitive to high-temperature and UV-A treatment [3,37,38,65]. Recent investigations showed that the alteration in organization of the LHCII-PSII supercomplex, i.e., the amount and oligomerization of the LHCII, which strongly modifies the electric properties of the thylakoid membranes, influences both the energy redistribution between the two photosystems and the oxygen production reaction [13]. Shutilova et al. [75] supposed that the decreased content of LHCII leads to destabilization of the OEC and decreases the thermostability of the photosynthetic membranes. Taking into account the results obtained for the influence of the UV-A and high temperature on the thylakoid membranes of pea mutants (Figure 23.2 and Table 23.2), greening plants, and the *Chlorina f2* barley mutant (Figure 23.1 and Table 23.1), it can be suggested that the organization of the LHCII-PSII supercomplex is determinant for different sensitivity of the photosynthetic apparatus to these stress factors. The susceptibility of the energy transfer and the oxygen evolution to UV-A radiation and high temperatures decreases with the increase in the amount and oligomerization of LHCII after the treatment of isolated thylakoid membranes. From the results reviewed in this study, it can be concluded that the oligomeric forms of LHCII play a key role for the sensitivity of the photosynthetic apparatus to temperature treatment and UV-A radiation.

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# *Part IV*

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## *Plant and Crop Responses to Pollution Stress*

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# 24 Plant Responses to Toxic Metal Stress\*

*Elena Masarovičová, Katarína Král'ová,  
and František Šeršeň*

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## 24.1 INTRODUCTION

Plants play an ever-increasing role in providing safe and healthy food for a growing world population, and in replacing the limited and expensive fossil resources as feedstock for the production of energy and industrial materials toward environmental protection. The transition to a sustainable economy is based largely on the renewable biological resources—the “knowledge-based bio-economy,” which is inevitable and desirable. The strategic agenda for plant research outlines an approach that can contribute toward addressing major socioeconomic challenges: (a) To fulfill the consumer demand for safe, sustainable, and healthy food. Novel plants aim at delivering nonallergic foods and foods with longer shelf lives, better nutritional composition, and more varied tastes. (b) To increase the agricultural productivity while decreasing its environmental footprint. Novel plants may need fewer inputs in terms of water, fertilizer, or pesticides and will be more stress resistant, for instance, against drought or seasonal instabilities that are caused by climate change. (c) To exploit the potential of biomass for the production of industrial materials. Plants (crops and trees)

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\* This chapter is sincerely dedicated to the memory of Dr. Zdeněk Šesták (1932–2008), long-time editor in chief of *Photosynthetica* and Dr. Jiří Čatský (1932–2008), long-time editor in chief of *Biologia Plantarum*.

and plant waste have become important sources for the production of energy, biofuels, and biopolymers by replacing the use of fossil fuels as feedstock. The production of novel, high value-added materials will be possible in “plant factories.” However, new technologies must be applied within the system that are both economically and environmentally sustainable. Advances in plant genomics and biotechnology can help Europe to address challenges, for instance, with stress-resistant plants. There are three priorities: (a) to produce more affordable, healthy, and better quality of food products; (b) to encourage environmental and agricultural sustainability; and (c) to enhance competitiveness in European agriculture, forestry, and industry. The European Union Commissioner for Science and Research, Janez Potočnik, outlined this approach in his presentation related to the strategic research agenda for the “Plants for the Future” technology platform [1]. It should be stressed that the negative effects of toxic metals on crops are manifested not only in the undesirable decrease of crop yield but also in the endangered food safety, causing serious action and intervention into the whole human population (cf. [2–5]).

## 24.2 CLASSIFICATION OF METAL IONS ACCORDING TO THEIR TOXICITY

Nieboer and Richardson [6] classified metals based on their ionic and covalent bonding tendencies, and donor-atom preference of metals. In this classification, two indices were used: (a) index  $(\chi_m)^2r$  that reflects the relative ability of the metal to form covalent *versus* ionic bonds and (b) index  $z^2/r$  expressing an effective measure of ionic interactions (where  $z$  is the ion charge,  $\chi_m$  is the Pauling electronegativity, and  $r$  is the ionic radius corresponding to the most common coordination number). These authors used the dependence of the covalent index  $(\chi_m)^2r$  *versus* the ionic  $z^2/r$  index as a base for the classification of metal and metalloid ions to three classes. Metals of **class A** are oxygen donor-atom seekers, whereas those of **class B** are nitrogen and sulfur seekers, and metals that ranged to the borderline metals are characterized by ambivalent affinity for all three donor-atoms. In general, for a fixed value of the ionic index, the toxicity increases with an increasing magnitude of the covalent index; conversely, for a fixed value of the covalent index, toxicity increases with increasing magnitude of the ionic index [7].

In Pearson's classification [8], metal acceptors and the ligand donors are divided into “hard and soft categories.” A **hard acceptor** is characterized by low polarizability, low electronegativity, and large positive charge density; whereas a hard donor by low electron mobility or polarizability, but high electronegativity and high negative charge density. The characteristics for **soft donors and acceptors** are opposite. According to hard soft acids and bases (HSAB), the hard acceptors prefer to bind hard donors and soft acceptors prefer to bind soft donors to form stable compounds [9]. **Class A** (hard) metals are Lewis acids (electron acceptors) of small size and low polarizability (deformability of the electron sheath or hardness), e.g., Na, Mg, Al, and K. On the other hand, **class B** (soft) metals are Lewis acids (electron acceptors) of large size and high polarizability (softness): Cu(I), Pd, Ag, Cd, Ir, Pt, Au, Hg, Ti, and Pb(II), and to the borderline (intermediate) metals belong V, Cr, Mn, Fe(II), Co, Ni, Cu(II), Zn, Rh, Pb(IV), and Sn [10].

## 24.3 EFFECT OF TOXIC METALS ON PHOTOSYNTHETIC ELECTRON TRANSPORT

Light reactions in photosynthesis are closely connected with the pigment excitation in the antenna system and subsequent electron transport through photosynthetic apparatus, water decomposition, and finally with oxygen release. Photosynthetic electron transport (PET) can be investigated by several methods in intact preparations (plant leaves, algae, and cyanophytes), in plant chloroplasts (intact chloroplasts or chloroplasts without outer membrane), or in experimentally prepared parts of the photosynthetic apparatus (photosystem (PS) I or PS II). For the PET study in intact samples, the determination of oxygen or CO<sub>2</sub> concentrations in the atmosphere or in water (in the closed space

surrounding the sample) or the measurement of chlorophyll fluorescence are used. PET in chloroplasts can be estimated by several methods:

1. By electrochemical measurements of oxygen concentration using the Clark electrode [11] the PET through the whole photosynthetic apparatus is registered.
2. By spectrophotometric methods that can be used for monitoring of the PET through individual parts of the photosynthetic apparatus. The Hill reaction is defined as the photoreduction of an electron acceptor (e.g., 2,6-dichlorophenol-indophenol [DCPIP] or a ferric salt) by the hydrogens of water that are connected with the evolution of oxygen occurring in the chloroplast suspension [12]. Due to the irradiation of the chloroplasts, the photoreduction of the artificial electron acceptor DCPIP to DCPIPH<sub>2</sub> occurs and is connected with the decolorization of the initially purple-colored suspension. By measuring absorbance at 600nm, the rate of DCPIP photoreduction and the rate of the water decomposition can be determined. Because the site of the DCPIP action is plastoquinone pool (PQ) on the acceptor side of PS II [13], this method is suitable for the PET monitoring through PS II. The rate of PET through PS I can be followed spectrophotometrically by DCPIPH<sub>2</sub> photooxidation [14]; however, it is necessary to secure the PET interruption between PS II and PS I of chloroplasts (for this purpose usually the DCMU is used because its site of action is situated in Q<sub>B</sub> on the acceptor side of PS II). The colorless DCPIPH<sub>2</sub> prepared by using the ascorbate serves as an artificial electron donor in the site of the plastocyanin on the donor side of PS I [15]. After photooxidation of DCPIPH<sub>2</sub>, the suspension will be purple-colored. The site of action of PET inhibitors can be more closely specified by the use of chlorophyll fluorescence (e.g., [16]) or by electron paramagnetic resonance (EPR). In the following subhead, we will focus our attention on the EPR method.

EPR is a very suitable method for investigating the effects of the transition metal ions on the photosynthetic apparatus due to two reasons:

1. Intact chloroplasts of algae and vascular plants exhibit EPR signals in the region of free radicals ( $g = 2.00$ ), which are stable during several hours [17] and could be registered at laboratory temperature by the conventional continual wave EPR apparatus. For the first time these signals were observed by Commoner et al. [18]. These signals were later denoted as signal I ( $g = 2.0026$ ,  $\Delta B_{pp} = 0.8$  mT) and signal II ( $g = 2.0046$ ,  $\Delta B_{pp} = 2$  mT) indicating their connection with the photosystem (PS) I and PS II, respectively [19]. Signal II consists of two components, namely, signal II<sub>slow</sub>, which is observable in the dark and signal II<sub>very fast</sub>, which occurs at the irradiation of the chloroplasts by the visible light and represents the intensity increase of signal II at the irradiation of chloroplasts by the visible light. It was found that signal II<sub>slow</sub> belongs to the intermediate D<sup>•</sup> and a signal II<sub>very fast</sub> belongs to the intermediate Z<sup>•</sup>. Intermediates Z<sup>•</sup> and D<sup>•</sup> are tyrosine radicals, which are situated at the 161st position in D<sub>1</sub> and D<sub>2</sub> proteins that are situated on the donor side of the PS II [20]. The EPR signal I is associated with the cation-radical of the chlorophyll dimmer situated in the core of PS I [17].
2. It is widely known that some ions of transition metals (Cu<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>, etc.) have unpaired spins and thus they are paramagnetic and will exhibit characteristic EPR spectra depending on the properties of the ligand. Addition of transition metal compounds to chloroplasts results in the interaction with some amino acid residues of the photosynthetic proteins that are reflected in the changes in EPR spectra of these ions [21].

### 24.3.1 COPPER

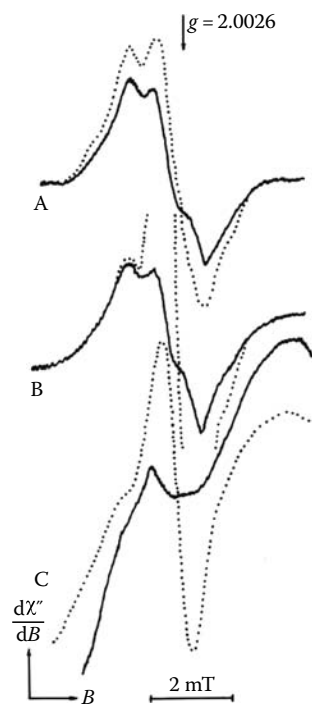
Copper belongs to transition metals that exhibit extremely toxic effects on the photosynthetic apparatus of algae and vascular plants whereby several sites of Cu<sup>2+</sup> action in the PET chain were determined [22–25]. Some authors placed the site of Cu<sup>2+</sup> action in PS I without a precise localization

on a certain member of the photosynthetic chain [26–28], others situated them in ferredoxine, that is, on the acceptor side of PS I [29] or in cytochrome *b* on the donor side of PS I [30]. Similarly, several authors located the site of  $\text{Cu}^{2+}$  action on both sides of PS II [26,31,32]. The donor side of PS II without the precise specification of the site of  $\text{Cu}^{2+}$  action was assumed by Cedeno-Maldonado et al. [33], Bohner et al. [28], Samuelsson and Öquist [27], and Samson et al. [34]. On the other hand, individual authors have determined the precise site for the action of the  $\text{Cu}^{2+}$  ions as follows:

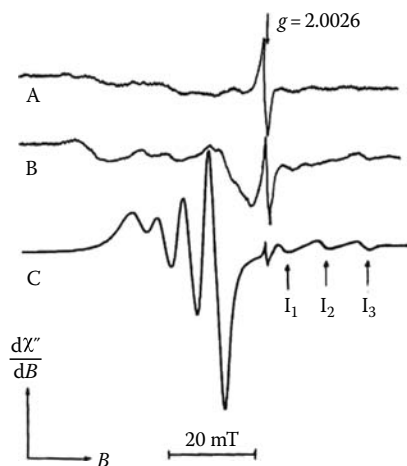
- Directly in protein  $\text{D}_1$  [35]
- Between  $\text{Tyr}_Z$  and P 680 [29,36–39]
- Directly in  $\text{Tyr}_Z$  [40]
- In both intermediates ( $\text{Tyr}_Z$  and  $\text{Tyr}_D$ ) [41,42]
- In the manganese cluster on the donor side of PS II [41,42]
- In  $\text{Q}_A$  and  $\text{Q}_B$  on the acceptor side of PS II [43–48]
- On both sides of PS II, namely in  $\text{Tyr}_Z$  and in  $\text{Q}_A$  and  $\text{Q}_B$ , respectively [49,50]
- In nonheme  $\text{Fe}^{2+}$  in PS II (causing displacement of iron ions [51])
- On cytochrome  $b_{559}$  in the photosystem II thylakoids [52,53]
- In the antenna chlorophyll of PS II [54]

Moreover,  $\text{Cu}^{2+}$  ions interact directly with the thylakoid membranes of the photosynthetic apparatus [55] and induce an alteration in the lipid composition, that is, they decreased the molar ratio of the monogalactosyldiacylglycerol to digalactosyldiacylglycerol in PS II [56].

Effect of  $\text{Cu}^{2+}$  ions on the photosynthetic apparatus using  $\text{Cu(II)}$  compounds with different ligands was studied by EPR spectroscopy. It was found that  $\text{Cu}^{2+}$  ions interact with Z/D intermediates or with amino acids constituting photosynthetic peptides  $\text{D}_1$  and  $\text{D}_2$ , and so the oxidation of Z/D could not occur [21,41,57]. This fact was demonstrated by a decrease of the intensity of both the EPR signals, that is, signal  $\text{II}_{\text{slow}}$  and  $\text{II}_{\text{very fast}}$  in  $\text{Cu(II)}$ -treated chloroplasts. Figure 24.1 shows EPR spectra of the untreated chloroplast suspension as well as those in the presence of  $\text{Cu}^{2+}$  in the dark (full lines) and in the light (dotted lines). The EPR signal at  $g = 2.0046$  and line width  $\Delta B_{\text{pp}} \sim 2 \text{ mT}$  belongs almost completely to signal  $\text{II}_{\text{slow}}$  (Figure 24.1A, full line), whereas the EPR signal induced by light corresponds approximately to signal  $\text{II}_{\text{very fast}}$  (Figure 24.1A, the difference between the light and dark spectra). From Figure 24.1C (full line) it is evident that due to the effect of  $\text{Cu}^{2+}$ , the EPR signals  $\text{II}_{\text{slow}}$  and  $\text{II}_{\text{very fast}}$  completely disappeared. Moreover, in the presence of  $\text{Cu}^{2+}$  ions, a great increase of signal I of the chloroplasts at  $g = 2.0026$  and width  $\Delta B_{\text{pp}} = 0.7 \text{ mT}$  was observed (Figure 24.1C, dotted line). This pronounced increase of EPR signal I in the light belonging to the cation radical of the chlorophyll dimmer situated in the core of PS I (P700) [17] in the presence of  $\text{Cu}^{2+}$  ions can be explained as follows: P700, which is excited in the light, easily oxidizes due to the interaction with the primary acceptor of PS I. However, in undamaged chloroplasts its reduction also occurs simultaneously due to the supply of electrons from PS II. If the reduction of P700 is eliminated, that is, PET is disrupted, P700 remains in the oxidized state what is reflected in the intense increase of signal I in the light. Thus, it can be concluded that P700



**FIGURE 24.1** The EPR spectra of untreated spinach chloroplasts (A) and chloroplasts with  $10 \text{ mol m}^{-3}$  (B) and  $50 \text{ mol m}^{-3}$  (C) of  $\text{Cu(pyr-}\beta\text{-ala)}$ . The full line were recorded in the dark, and the dotted ones were recorded in the light. The dotted lines in C was recorded at 0.5 amplification against other lines. B is magnetic induction given in millitesla (mT), and  $d\chi''/dB$  is the first derivative of the imaginary part of magnetic susceptibility  $\chi$  with respect to B. (From Šeršef, F. et al., *J. Plant Physiol.*, 151, 299, 1997. With permission.)



**FIGURE 24.2** The EPR spectra of untreated chloroplasts (A) and that of copper (II) present in the resuspended sediment of centrifuged chloroplasts (10 min at 15,000 g at 4°C) treated with  $10 \text{ mol m}^{-3}$   $\text{Cu(pyr-}\beta\text{-ala)}$  (B) and that of  $\text{Cu(pyr-}\beta\text{-ala)}$  in chloroplasts suspension before centrifugation (C). (From Šeršeň, F. et al., *J. Plant Physiol.*, 151, 299, 1997. With permission.)

was not impaired by diaqua-(N-pyruvidene- $\beta$ -alaninato) copper(II) monohydrate ( $\text{Cu(pyr-}\beta\text{-ala)}$ ). Similarly, Schröder et al. [40] and Jegershöld et al. [50] indicated that  $\text{Cu}^{2+}$  ions damage exclusively the intermediate Z; however, they treated the chloroplasts only at very low  $\text{Cu}^{2+}$  concentrations.

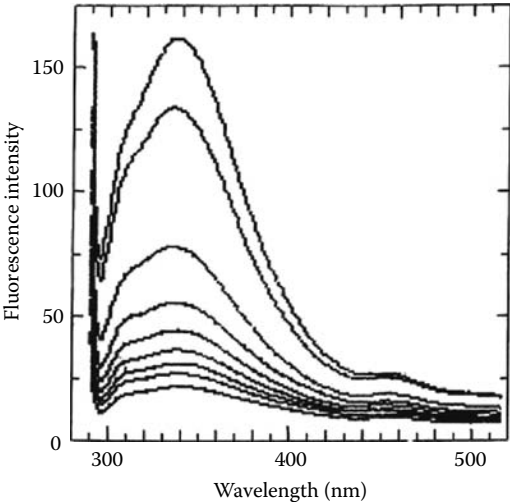
In addition, in the presence of  $\text{Cu}^{2+}$  ions in the EPR spectrum of chloroplasts, the appearance of characteristic lines belonging to the  $\text{Mn}^{2+}$  ions can be observed (Figure 24.2). In this spectrum, only three from six lines corresponding to free  $\text{Mn}^{2+}$  ions are resolved (marked with arrows  $I_1$ ,  $I_2$ , and  $I_3$  on line C), the other three lines remain unresolved due to high signal intensity of  $\text{Cu}^{2+}$  ions.

The appearance of the characteristic lines belonging to the  $\text{Mn}^{2+}$  ions confirm that the  $\text{Cu}^{2+}$  ion interacts also with the manganese cluster that is a component of the oxygen evolving complex situated in the donor side of PS II. Due to this interaction,  $\text{Cu}^{2+}$  ions release  $\text{Mn}^{2+}$  ions from the manganese cluster into interior of the thylakoid membrane [41,58], and free  $\text{Mn}^{2+}$  ions can be easily detected by EPR spectroscopy (for example, see Figure 24.6). The loss of manganese ions from PS II was observed also by Jegershöld et al. [50].

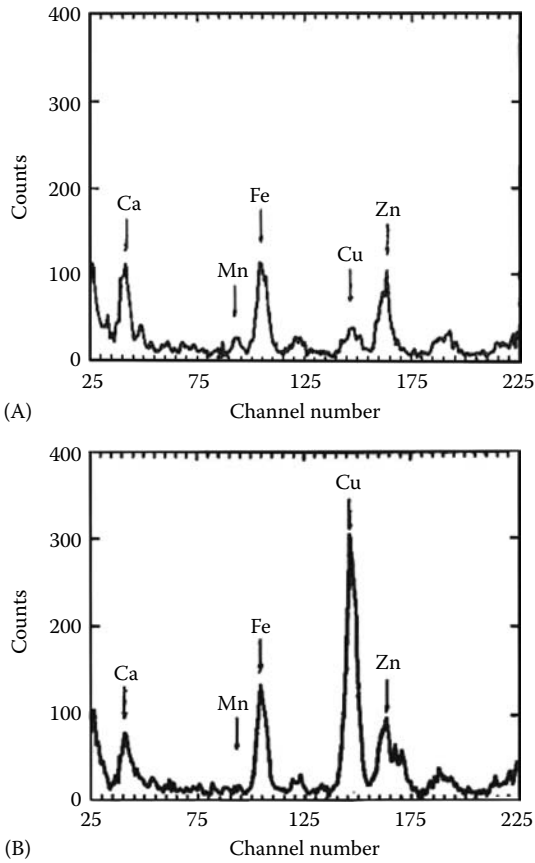
The results related to the determined copper amount in the sediment and supernatant after centrifugation of  $\text{Cu(pyr-}\beta\text{-ala)}$ -treated chloroplasts indicate that the studied  $\text{Cu(II)}$  complex is able to interact not only with  $D_1$  and  $D_2$  proteins, but also with some other proteins that are present in the photosynthetic centers. Using radionuclide fluorescence analysis (RFA), it was found that the number of copper ions bound to one set of PS I and PS II in the complex-treated chloroplasts is greater than the sum of bivalent ions ( $\text{Cu}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mn}^{2+}$ ) that are present in untreated chloroplasts. Consequently,  $\text{Cu}^{2+}$  ions from  $\text{Cu(pyr-}\beta\text{-ala)}$  must also be bound to the proteins of photosynthetic centers whereby  $\text{Cu(pyr-}\beta\text{-ala)}$  ligands will be substituted by amino acids of these proteins. The interaction of  $\text{Cu(pyr-}\beta\text{-ala)}$  with aromatic amino acids was also confirmed by the quenching of the fluorescence band at 332 nm in  $\text{Cu(pyr-}\beta\text{-ala)}$ -treated chloroplasts (Figure 24.3).

The formation of the chelate-bound copper in the chloroplasts is reflected in the changed shape of the EPR signals of  $\text{Cu}^{2+}$  (Figure 24.2, compare lines B and C). It can be assumed that by binding the  $\text{Cu}^{2+}$  ions to the voluminous protein molecules, the motion of the spin of the  $\text{Cu}^{2+}$  electron will be restricted, resulting in the diminution of the hyperfine structure in its EPR spectrum (Figure 24.2, line B). In addition to the above-discussed mechanism of  $\text{Cu(pyr-}\beta\text{-ala)}$  action, it can be assumed that  $\text{Cu}^{2+}$  can replace other bivalent ions ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ) located in the photosynthetic centers (Figure 24.4).

The above discussed effects of  $\text{Cu(pyr-}\beta\text{-ala)}$  on the photosynthetic apparatus of spinach chloroplasts were also found for a set of  $\text{Cu(II)}$  compounds with biologically active ligands using freshwater



**FIGURE 24.3** The fluorescence emission spectra of aromatic amino acids contained in untreated chloroplasts and in chloroplasts treated with 0.04, 0.138, 0.253, 0.48, 0.539, 0.619, and 0.791 mol m<sup>-3</sup> of Cu(pyr-β-ala) (the curves from top to bottom). The excitation wavelength was 275 nm. (From Šeršeň, F. et al., *J. Plant Physiol.*, 151, 299, 1997. With permission.)



**FIGURE 24.4** The RFA spectrum of untreated chloroplasts (A) and that of chloroplasts treated with Cu(pyr-β-ala) (B). (From Šeršeň, F. et al., *J. Plant Physiol.*, 151, 299, 1997. With permission.)



algae *Chlorella vulgaris* [42,57]. However, it could be stressed that the manganese cluster in algae was damaged only by diaqua(4-chloro-2-methylphenoxyacetato)copper(II) complex [42] and not by copper(II) complexes with ligands having anti-inflammatory activity (e.g., flufenamate, niflumate, and naproxenate) [57].

In general, it can be concluded that the intensity of the inhibitory action of  $\text{Cu}^{2+}$  ions is closely connected with the stability constants of copper compounds and it was shown that the different coordinating modes of acidoligands strongly affected the biological activity of Cu(II) compounds related to PET in spinach chloroplasts [59,60]. The simple carboxylatocopper(II) complexes of the general formula  $\text{Cu}(\text{RCOO})_2 \cdot n\text{H}_2\text{O}$  have been one or two orders more effective inhibitors of photosynthesis than complex cuprates with Cu–NCO(S)–Cu' bridges within the dimeric  $[\text{Cu}_2(\text{TSB})_2\text{X}_2]$  unit containing tridentate Schiff base ( $\text{TSB}^{2-}$ ) of N-salicylideneaminoacide type and pseudohalogenide (X) ligands [60].

### 24.3.2 MERCURY

Similarly to the effects of copper, several sites of action on the photosynthetic apparatus were also found for mercury. Some researchers situated the site of action of  $\text{Hg}^{2+}$  ions at the donor side of PS I:

- Without precise specification [61]
- At the site of plastocyanin [62–64]
- At the acceptor side of PS I, either at ferredoxin action site [65,66] or in the  $\text{F}_\text{B}$  iron-sulfur
- Cluster [67,68]

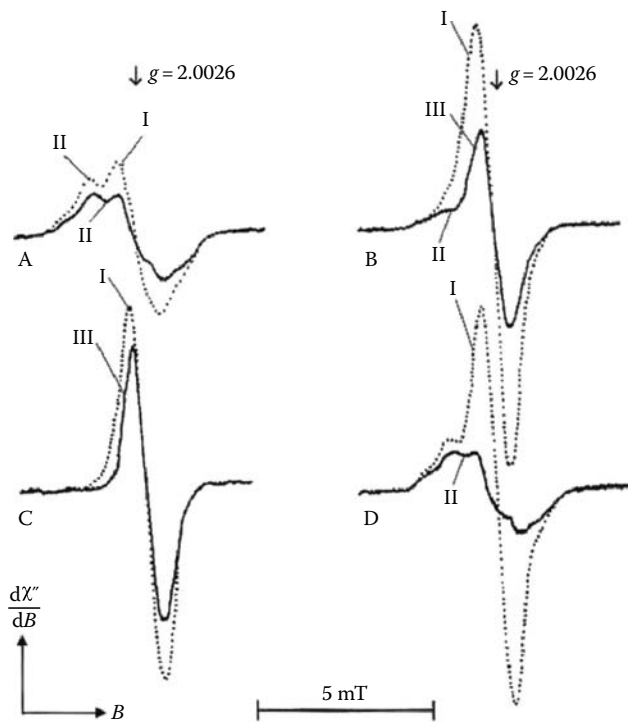
Other authors localize the site of  $\text{Hg}^{2+}$  action in the PS II:

- Without exact determination of the site of action [62,64,65]
- On the donor side of PS II, namely in the oxygen evolving complex (OEC) [30,66,69–71]
- Directly in the core of PS II [72]
- On the acceptor side of PS II between the  $\text{Q}_\text{A}$  and  $\text{Q}_\text{B}$  quinones [73–75]

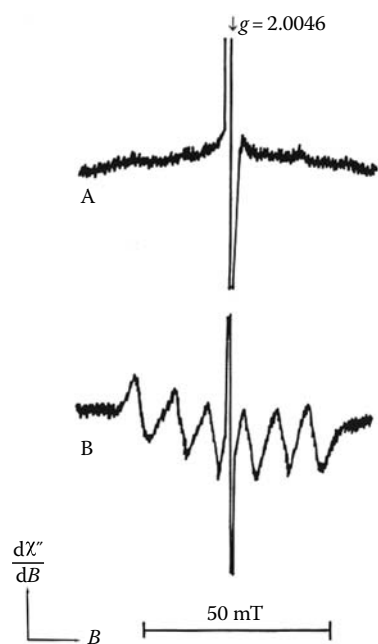
Moreover, it was found that  $\text{Hg}^{2+}$  ions interacted with phycobilisomes that form a part of the light-harvesting complex of *Spirulina platensis* [72,76,77], with the light-harvesting complex of PS II in the spinach chloroplasts [78,79] and that they also inhibit the formation of ATP [30,65,66]. Several authors have explained the mechanisms of  $\text{Hg}^{2+}$  action by the formation of complexes with amino acids in the proteins of photosynthetic centers due to a strong affinity of  $\text{Hg}^{2+}$  ions to C=O, C–N, C–S, and C–SH groups [71,78,79].

EPR spectra of chloroplasts treated with  $\text{Hg}^{2+}$  ions exhibit marked changes against the untreated ones [80,81]. First, the decreasing signal intensities of both the signals  $\text{II}_{\text{slow}}$  (line II in Figure 24.5B,C) and  $\text{II}_{\text{very fast}}$  (abstraction of lines II in the light and in the dark, Figure 24.5B,C). It clearly illustrates the interaction of the  $\text{Hg}^{2+}$  ions with  $\text{Z}'/\text{D}'$  intermediates or with a part of protein in their near vicinity. Hence, the oxidation of the Z/D tyrosines cannot occur and PET between PS II and PS I will be interrupted (which was confirmed also by the increase of EPR signal I intensity in the light) (lines I in Figure 24.5B,C).

On the other hand, the increase of the signal I intensity in the dark is connected with the damage of PS I in  $\text{Hg}$ -treated chloroplasts (line III in Figure 24.5B,C, full lines) due to oxidation of the chlorophyll dimmer in the core of PS I. The toxic effect of  $\text{Hg}^{2+}$  on the photosynthetic apparatus of chloroplasts was also manifested by the release of  $\text{Mn}^{2+}$  ions from manganese cluster into the interior of thylakoid membranes (Figure 24.6). The binding of  $\text{Hg}^{2+}$  ions to photosynthetic proteins was confirmed by the RFA method and by the fluorescence of aromatic amino acids [80].



**FIGURE 24.5** EPR spectra of untreated spinach chloroplasts (A) and chloroplasts treated with 8 mol m<sup>-3</sup> of HgCl<sub>2</sub> (B) or 40 mol m<sup>-3</sup> of HgCl<sub>2</sub> (C) or with 5 mol m<sup>-3</sup> DCMU (D). The full line spectra were recorded in the dark and the dotted ones were recorded in the light. (From Šeršeň, F. et al., *Photosynthetica*, 35, 551, 1998. With permission.)



**FIGURE 24.6** EPR spectra of Mn<sup>2+</sup> ions in untreated chloroplasts and in chloroplasts treated with 50 mol m<sup>-3</sup> HgCl<sub>2</sub> (B). (From Šeršeň, F. et al., *Photosynthetica*, 35, 551, 1998. With permission.)

### 24.3.3 ORGANOMETALLIC COMPOUNDS

Organomercuric compounds (methylmercuric chloride, phenylmercuric acetate, and phenylmercuric borate) were found to inhibit PET in spinach chloroplasts with lower effectiveness than  $\text{HgCl}_2$ . EPR spectroscopy suggested that these compounds—in contrast to  $\text{HgCl}_2$  action—do not interact with the intermediates  $\text{Z}'/\text{D}^{\bullet}$  and with the manganese cluster. However, organomercuric compounds interacted with PS I [82]. It can be assumed that their site of action is ferredoxine situated on the acceptor side of PS I [65].

The model tributyltin compound, tributyltin naphthenate, inhibited PET in spinach chloroplasts. The site of its inhibitory action was found to be  $\text{Z}'/\text{D}^{\bullet}$  intermediates or their vicinity as well as manganese cluster in the oxygen-evolving complex and PS I. The mechanism of the inhibitory action is probably connected with the interaction between the tributyltin naphthenate and amino acids in photosynthetic proteins as confirmed by fluorescence measurements [83].

### 24.3.4 CADMIUM

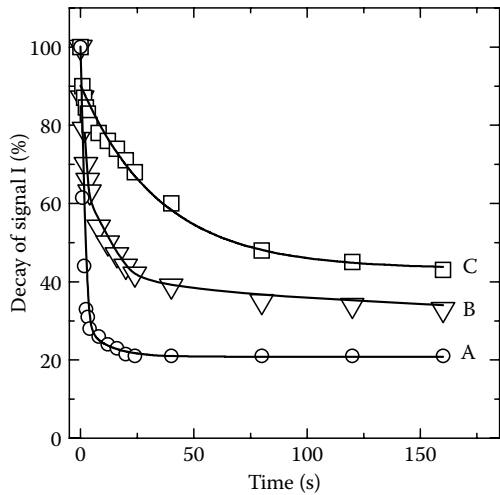
Cadmium belongs to the class of major environmental pollutants with toxic effects on the vascular plants that are caused by direct and indirect mechanisms of its action on the photosynthetic apparatus [84]. For  $\text{Cd}^{2+}$  ions, the following sites of action were suggested:

- The photosystem (PS) II without precise localization [85–87]
- The donor side of the PS II, particularly in the oxygen evolving complex or in its vicinity [66,88–95]
- $\text{Z}/\text{D}$  intermediates [93,94]
- The site of  $\text{Q}_\text{A}$  or  $\text{Q}_\text{B}$  on the acceptor side of PS II [30,93,96]

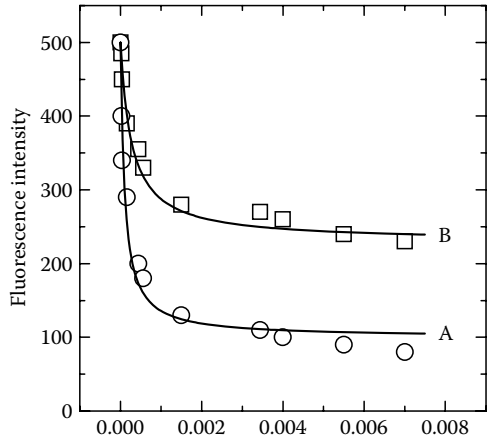
Several authors have reported low sensitivity of PS I activity or its inactiveness to  $\text{Cd}^{2+}$  ions [87–90,97]. In hydroponically cultivated *Spinacia oleracea* L. plants, Fagioni et al. [98] observed high sensitivity of PS I to Cd and only minor effects on PS II. Besides the above-mentioned effects,  $\text{Cd}^{2+}$  ions cause inhibition of ATP [66] and chlorophyll synthesis [95,98,99], disorganization of thylakoid membranes [100,101], and changes in the lipid composition of thylakoid membranes [102].

$\text{Cd}^{2+}$  is known to replace  $\text{Ca}^{2+}$  ions in the Ca/Mn cluster that constitutes the oxygen-evolving center [92,93,103–105]. Sigfridson et al. [93] using the flash-induced variable fluorescence and EPR spectroscopy observed the following effects of  $\text{Cd}^{2+}$  ions on the photosynthetic apparatus: loss of electron transfer from intermediates  $\text{Z}/\text{D}$  to  $\text{P-680}^{++}$ ; slowdown of electron transfer between  $\text{Q}_\text{A}^{\bullet-}$  to  $\text{Q}_\text{B}$  and inhibition of steady state oxygen evolution.

Using EPR spectroscopy Šeršeň and Král'ová [94] found that  $\text{Cd}^{2+}$  ions interact with  $\text{Z}'/\text{D}^{\bullet}$  intermediates (or their near vicinity) and with the manganese cluster in OEC that resulted in the release of  $\text{Mn}^{2+}$  ions into the interior of thylakoid membranes. It was confirmed that  $\text{Cd}^{2+}$  ions interact also with the primary donor of PS I (P700) whereby the chlorophyll *a* dimmer in the core of PS I is oxidized also in dark. In addition, the deleterious direct, cyclic, and noncyclic electron flow through PSI was observed and was confirmed by the kinetics of EPR signal I decay after switching-off of the light (Figure 24.7). From this figure it is evident that in the control sample, where all mechanisms (direct, cyclic, and noncyclic) of reduction of  $\text{P700}^+$  are not damaged, the decrease of the signal I intensity to the original value after the switch-off of the light is very fast and is in the range of 5 s (Figure 24.7, line A). As the control sample, chloroplasts treated with DCMU were used, which caused complete inhibition of the electron flow from PS II to PS I without damage of the PS I. After the addition of  $\text{CdCl}_2$  or  $\text{HgCl}_2$  to chloroplast suspension, the rate of the decrease of signal I intensity was restricted (Figure 24.7 lines B,C) and indicated that all reduction mechanisms of  $\text{P700}^+$  were damaged by the applied metal chlorides; however, the  $\text{HgCl}_2$  action was more effective.



**FIGURE 24.7** The time dependence of EPR signal I intensity after switching off light in chloroplasts treated with 5 mM DCMU (A), 0.05 M CdCl<sub>2</sub> (B), and 0.05 M HgCl<sub>2</sub> (C). (From Šeršeň, F. and Král'ová, K., *Photosynthetica*, 39, 575, 2001. With permission.)



**FIGURE 24.8** The dependencies of fluorescence intensity of aromatic amino acid residues in chloroplast peptides on concentrations of HgCl<sub>2</sub> (A) and CdCl<sub>2</sub> (B). (From Šeršeň, F. and Král'ová, K., *Photosynthetica*, 39, 575, 2001. With permission.)

Šeršeň and Král'ová [94] using fluorescence spectroscopy demonstrated the interaction of CdCl<sub>2</sub> with the aromatic amino acid residues in photosynthetic proteins. From Figure 24.8 it is evident that with increasing CdCl<sub>2</sub> concentration the intensity of the fluorescence emission band at 334 nm shows a decrease. The decay of this emission band belonging mainly to the tryptophan residues [106] is caused by the formation of complexes between cadmium ions and aromatic amino acids contained in the peptides of the photosynthetic apparatus. The quenching of the emission of aromatic amino acids was observed also in chloroplasts treated with HgCl<sub>2</sub> [80,81]. According to the procedure published in the work of Tominaga et al. [107], the equilibrium constants (*K*) were calculated from the concentration dependence of the intensity of fluorescence band at 334 nm. The *K* values obtained by computer fitting of the quenching curves A and B (Figure 24.8), were *K* = 10200 (*r*<sup>2</sup> = 0.97) for HgCl<sub>2</sub> and *K* = 3700 (*r*<sup>2</sup> = 0.97) for CdCl<sub>2</sub>.

Based on this finding, it could be concluded that the higher inhibitory efficiency of  $\text{HgCl}_2$  with respect to that of  $\text{CdCl}_2$  is caused by the higher ability of mercury ions to form complexes with aromatic amino acids [94]. Similarly, using the fluorescence method, it was found that the stability constants of the formation of complexes between tryptophan and heavy metals decreased in the following order:  $3.60 \times 10^8 \text{ dm}^3 \text{ mol}^{-1}$  for  $\text{Cu}^{2+}$ ,  $1.82 \times 10^5 \text{ dm}^3 \text{ mol}^{-1}$  for  $\text{Hg}^{2+}$ ,  $1.70 \times 10^4 \text{ dm}^3 \text{ mol}^{-1}$  for  $\text{Cd}^{2+}$ , and  $3.72 \times 10^3 \text{ dm}^3 \text{ mol}^{-1}$  for  $\text{Ni}^{2+}$ . The determined stability constants of the tryptophan–heavy metal complexes showed close correlation with the inhibitory effect of corresponding metals on PET [108].

#### 24.3.5 NICKEL

Krupa et al. [109] investigated the effect of *in vivo*  $\text{Ni}^{2+}$  toxicity on the photosynthetic system of primary bean leaves. The results of these authors indicated an indirect effect of nickel on photosystems related to the disturbances caused by the metal in the Calvin cycle reactions and down-regulation or even feedback inhibition of electron transport by the excessive amounts of ATP and NADPH accumulated due to nonefficient dark reactions.

$\text{Ni(II)}$  complexes with N-donor ligands affected PET through the photosynthetic apparatus due to interaction with  $\text{Z}'/\text{D}^*$  intermediates and manganese cluster in OEC [110]. It was shown that  $\text{Ni}^{2+}$  strongly inhibits oxygen evolution in the millimolar range of concentration, and the extrinsic polypeptides of 16 and 24 kDa associated with the oxygen-evolving complex of photosystem II were depleted following  $\text{Ni}^{2+}$  treatment. Consequently, it was deduced that interaction of  $\text{Ni}^{2+}$  with these polypeptides caused a conformational change that induced their release together with  $\text{Ca}^{2+}$  from the oxygen-evolving complex of PS II with consecutive inhibition of the electron transport activity [111]. Similarly, in *Chlamydomonas reinhardtii* algae, the inhibitory effect of  $\text{Ni}^{2+}$  on PET was reflected in the decline of oxygen evolution with increasing  $\text{Ni}^{2+}$  concentration. Nickel inactivated PS II activity; however, it did not affect the flow of electrons through PS I. The  $\text{Ni}^{2+}$  ions intensively inhibited photoreduction of 2,6-dichlorophenol-indophenol in broken *C. reinhardtii* cells and the activity could not be restored, not even by the addition of artificial electron donor 1,5-diphenylcarbazide. Thus, it can be assumed that nickel inhibited electron flow at the donor side of PS II [112]. The inhibitory effects of  $\text{Ni}^{2+}$  ions could be also connected with their ability to form complexes with amino acid residues in photosynthetic proteins [108].

#### 24.3.6 LEAD

Prasad et al. [113] found that PS II of *Nostoc muscorum* was more sensitive both to low and high concentrations of  $\text{Pb}^{2+}$  ions. A considerable inhibition of photosystem PS I was, however, observed at high concentrations only; an increase in Chl *a* fluorescence at high concentrations of Pb suggested that this metal inhibits the electron flow on the reducing side of the PS II reaction center. According to Kastori et al. [114],  $\text{Pb}^{2+}$ -treatment resulted not in the destruction but in the reduction of photosynthetic apparatus and decreased the efficiency of PS II electron transport. Reduction of the rate of whole chain electron transport, photochemical activities of PS II, and oxygen evolution due to  $\text{Pb}^{2+}$  treatments was confirmed also by Wu et al. [115], wherein the photoreduction activities of PS I were not changed. Ling and Hong [116] observed that  $\text{Pb}^{2+}$  ions accumulated in PS II of *Spirodela polyrrhiza* and damaged its secondary structure, decreased the absorbance of visible light, inhibited energy transfer among amino acids within the PS II protein-pigment complex, and reduced the energy transport from tyrosine residue to chlorophyll *a*.

#### 24.3.7 CHROMIUM

Experiments of Ali et al. [117] with *Lemna gibba* showed that chromium treatment resulted in the alteration of the PS II electron transport at both PS II oxidizing and reducing sides. Investigation of

fluorescence yields suggested for the site of Cr inhibitory effect the oxygen-evolving complex and  $Q_A$ . Those Cr-inhibitory effects were related to the change of the turnover of PS II D1 protein and the alteration of 24 and 33 kDa proteins of the OEC.

In *Spirodela polyrrhiza*, Cr affected several targets of PS II causing OEC damage and a decreased number of active reaction centers [118]. Bishnoi et al. [119] observed that in isolated chloroplasts exogenously added  $Cr^{6+}$  had little effect on the activity of PS II whereas in that of PS I it was markedly inhibited. In *Ocimum tenuiflorum* L., chromium induced lipid peroxidation coupled with potassium leakage and reductions in photosynthetic pigments, protein, cysteine, ascorbic acid, and non-protein thiol contents [120]. The overall photosynthetic efficiency of PS II and PS I of green fronds was found to be decreased after dichromate treatment [121]. According to Perreault et al. [122], dichromate has different sites of inhibition that are associated with photosystem II, photosystem I, and electron transport sink beyond photosystems. The chlorophyll protein complexes showed that the photosystem II (core complex as well as the connecting antenna) in *S. polyrrhiza* was more sensitive to chromate treatment than photosystem I and the peripheral light-harvesting complex of photosystem II [123].

### 24.3.8 ZINC

Rashid et al. [124] investigated the inhibitory effect of  $Zn^{2+}$  on PET and they reported the presence of an active  $Zn^{2+}$  inhibitory site on the donor side of the photosystem (PS) II. The authors assumed that elevated levels of  $Zn^{2+}$  strongly perturbed the conformation of the PS II core complex and might also affect the acceptor side of the photosystem. According to Jegerschöld et al. [51],  $Zn^{2+}$  caused the displacement of the nonheme  $Fe^{2+}$  in PS II. Rapid inactivation of electron transport at PS II due to treatment with  $Zn^{2+}$  in *Synechocystis aquatilis* f. *aquatilis* Sauvageau observed Chaloub et al. [125]. The authors indicated that electron transfer was inhibited at the reducing site of PS II. According to Tajmirriahi and Ahmed [126], Zn(II) ions interact with the light-harvesting proteins LHC-II of chloroplast thylakoid membranes, and C=O and C–N groups were the main coordination sites of  $Zn^{2+}$  at higher concentrations, whereas at low metal concentrations, binding with the protein carbonyl groups occurs.  $Zn^{2+}$  cations irreversibly suppressed the oxygen evolving function and produced disaggregation of the dimer structure of the OEC. Comparison of the effects of several heavy metal cations on the oxygen-evolving activity in isolated submembrane fragments enriched by PS II showed that the toxic action of metal cations increased in a sequence:  $Zn < Cd < Pb < Hg$  [127].

### 24.3.9 IRON

Harmful effect of iron excess on the PET was observed previously by several researchers (e.g., [128,129]). Mallic and Rai [128] observed the inhibition of photosynthesis and PET chain of *Anabaena doliolum* and *Chlorella vulgaris* by Fe and found that the mode of inhibition of the PET chain of both algae was similar; however, PS II showed greater sensitivity to iron. Kampfenkel et al. [129] observed that iron stress caused a 40% decrease of the photosynthetic rate in *Nicotiana plumbaginifolia* plants within 12h and the inhibition of photosynthesis was accompanied by an increased reduction of PS II.

Using EPR spectroscopy it was found that  $Fe^{3+}$  ions interacted with  $Z'/D^{\bullet}$  intermediates (or their near vicinity) located at the donor side of PS II and so the electron transport between the photosynthetic centers PS II and PS I was interrupted [130]. In addition, the treatment with  $[Fe(nia)_3(H_2O)_2](ClO_4)_3$  resulted in the release of Mn(II) from the oxygen evolving complex. In this iron compound, the  $ClO_4^-$  anions are not bound to the Fe atom by a coordination bond, and in the coordination sphere of  $[Fe(nia)_3(H_2O)_2](ClO_4)_3$  there are water molecules that can easily be substituted by another ligand, e.g., residues of amino acids in proteins [130].

## 24.4 RUBISCO ACTIVITY AND PROTEIN CONTENT UNDER METAL STRESS

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) catalyses  $\text{CO}_2$  assimilation and also functions as an oxygenase in the plants (see [131,132] for details). A prerequisite for the catalysis is an activation process, whereby an active site lysine is selectively carbamylated. The carbamyl group is then stabilized by a metal ion, which *in vivo* is  $\text{Mg}^{2+}$ . Other divalent metal ions can replace  $\text{Mg}^{2+}$  as activators *in vitro*, but the nature of the metal ion strongly influences the catalytic activity of the enzyme and has a differential effect on the ratio of the carboxylation reaction and the competing oxygenation reaction [133]. The deactivation of the enzyme after the substitution of  $\text{Mg}^{2+}$  in the ternary Rubisco complex by metal cations can result in loss of carboxylation capacity [134]. Rubisco is a hexameric enzyme that is composed of two subunits: a small subunit (SSU) encoded by a nuclear gene (*rbcS*), and a large subunit (LSU) encoded by a plastid gene (*rbcL*). Due to its high abundance, Rubisco represents an interesting target to express peptides or small proteins as fusion products at high levels [135]. Wang et al. [136] used spectral methods for the study of the mechanism of molecular interactions between  $\text{Pb}^{2+}$  and Rubisco. They found that the carboxylase activity of Rubisco gradually decreased with increasing concentrations of  $\text{Pb}^{2+}$  and that  $\text{Pb}^{2+}$  ions are directly bound to Rubisco. Based on the analysis of the inductively coupled plasma-mass spectrometry (ICP-MS) and the circular dichroism (CD) spectra, it was concluded that  $\text{Pb}^{2+}$  replaced  $\text{Mg}^{2+}$  from the catalytic center in Rubisco and that the binding of  $\text{Pb}^{2+}$  entirely altered its primary conformation, implying that the  $\text{Pb}^{2+}$  coordination created a new metal ion-active site form of Rubisco, thus leading to a reduction in its carboxylase activity. Xiao et al. [137] observed significant reduction of the activities of the key enzymes of carbon assimilation, especially Rubisco activase due to  $\text{Pb}^{2+}$  treatment in plants cultivated in the field experiments that were sprayed with various concentrations of  $\text{PbCl}_2$  solution. Lidon and Henriques [37] found that Rubisco activity in rice (*Oryza sativa* L.) plants cultivated in a nutrient solution showed a progressive decrease with increasing copper levels in the solution medium and even though excess copper affects the photosynthetic process in multiple ways, the prevailing effect is that on the activity of Rubisco, which in turn limits the overall photosynthetic activity.

Moustakas et al. [138] evaluated the effects of Cu and Pb on the activity of Rubisco in oat plants cultivated in the field and found that Cu and Pb application led to a pronounced reduction (47%) of chlorophyll *Chl* (*a* + *b*) content, accompanied by proportional changes in Rubisco activity. Similarly, Lidon and Henriques [37] stated that the prevailing toxic effect of copper and lead was on Rubisco activity, which in turn limited the overall photosynthetic activity. Lee and Roth [139] found that the application of  $5.0\text{ }\mu\text{M}$  Cd significantly reduced Rubisco activity. They also found that the activation and induction of Rubisco was inhibited by Cd and that the change in the levels of Rubisco activase leads to a subsequent alteration of Rubisco levels. Inhibition of Rubisco activity by toxic metals (other than the above-mentioned metals also Mn, Ni, and Zn) was confirmed in several plant species (see in [23]). Sulfhydryl group inactivation was suggested to explain the inhibitory effects of Pb, Cd, Zn, and Cu on the activity of chloroplast enzyme Rubisco and phosphoribulokinase *in vitro* [140].

The effect of air pollutants on forest woody plants manifests by visible or latent damage. Visible damage is the result of total collapse of the leaves or chronic damage caused by the sublethal substance concentrations throughout a long period. Under latent damage, the life functions of woody plants are negatively affected, especially the biochemical (enzymes and chloroplasts activities, protein, and chlorophyll contents) and physiological processes, e.g., photosynthesis that are manifested negatively in a lower biomass production (see [141] for details). Since harmful environmental impact on the activity of Rubisco and protein content in the leaves of broadleaf forest trees have not been investigated frequently, methodical approaches had to be modified [142]. We studied the effects of internal (ontogenetical development) and external factors (meteorological conditions and pollutants) on the above-mentioned biochemical features of three Slovak autochthonous oak species: *Quercus cerris* L., *Q. robur* L., and *Q. dalechampii* L. stemming from forest stands with different degrees

**TABLE 24.1**  
**Values of the Specific Rubisco Activity and Protein Content**  
**in the *Quercus dalechampii*, *Q. robur*, and *Q. cerris* Leaves**

| Specific Activity (Bq μg <sup>-1</sup> (Protein)) |                 |       | Protein Content (μg mm <sup>-3</sup> ) |                 |      |      |
|---------------------------------------------------|-----------------|-------|----------------------------------------|-----------------|------|------|
| Healthy Samples                                   | Damaged Samples |       | Healthy Samples                        | Damaged Samples |      |      |
|                                                   | A               | B     |                                        | A               | B    |      |
| <i>Q. dalechampii</i> :                           |                 |       |                                        |                 |      |      |
|                                                   | 58.55           | 37.42 | 29.96                                  | 7.20            | 1.92 | 0.29 |
|                                                   | 107.42          | 31.13 | 30.41                                  | 5.56            | 1.88 | 0.32 |
|                                                   | 97.22           | 27.78 | 20.01                                  | 2.38            | 1.88 | 0.45 |
|                                                   | 104.52          | —     | 19.34                                  | 3.01            | —    | 0.49 |
|                                                   |                 |       | 2.82                                   |                 |      |      |
| <i>x</i>                                          | 91.93           | 32.11 | 24.93                                  | 4.19            | 1.89 | 0.39 |
| <i>s<sub>x</sub></i>                              | 11.33           | 2.83  | 3.04                                   | 0.93            | 0.01 | 0.05 |
| <i>Q. robur</i> :                                 |                 |       |                                        |                 |      |      |
|                                                   | 41.98           | 25.41 | 20.10                                  | 6.90            | 1.16 | 0.45 |
|                                                   | 69.95           | 33.60 | 31.15                                  | 4.86            | 1.76 | 0.33 |
|                                                   | 83.77           | 17.78 | 28.87                                  | 4.38            | 1.16 | 0.39 |
|                                                   | —               | —     | 31.76                                  | 3.42            | —    | 0.49 |
|                                                   |                 |       | 25.39                                  |                 |      | 0.57 |
| <i>x</i>                                          | 65.23           | 25.60 | 27.45                                  | 4.89            | 1.36 | 0.45 |
| <i>s<sub>x</sub></i>                              | 12.29           | 4.57  | 2.15                                   | 0.73            | 0.20 | 0.04 |
| <i>Q. cerris</i> :                                |                 |       |                                        |                 |      |      |
|                                                   | 24.58           | 27.72 | 27.81                                  | 6.04            | 1.28 | 0.45 |
|                                                   | 34.63           | 20.78 | 30.53                                  | 4.47            | 1.36 | 0.73 |
|                                                   | 27.74           | 17.38 | 31.67                                  | 3.12            | 1.36 | 0.84 |
|                                                   | 26.57           | —     | 23.50                                  | 2.84            | —    | 0.77 |
|                                                   | —               | —     | 19.55                                  | 1.92            | —    | 0.77 |
| <i>x</i>                                          | 28.38           | 21.96 | 26.61                                  | 3.68            | 1.33 | 0.71 |
| <i>s<sub>x</sub></i>                              | 2.18            | 3.04  | 2.26                                   | 0.72            | 0.03 | 0.07 |

Source: Konečná, B. et al., *Photosynthetica*, 23, 566, 1989. With permission.  
Note: A, leaves of the seedlings transferred in the spring 1987 from damaged forest stand and transplanted into the garden; B, leaves of the seedlings that were processed immediately after sampling (July 1987); *x*, mean, *s<sub>x</sub>*, S.E.

of pollution damage. Both Rubisco activity and protein content in the oak leaves were significantly lowered (Table 24.1). The first species exhibited lower sensitivity of both biochemical characteristics to negative effects of the environment than *Q. robur* or *Q. dalechampii*. Overall, protein content was the most sensitive parameter contributing to the damage of oak leaves. It was found that in the damaged oak leaves, the protein content dropped the most among all the biochemical characteristics examined; thus *de novo* Rubisco synthesis was inhibited. In damaged leaves of all three oak species also a lower content of nonmetallic (K, Na, Ca, Mg, and S) and a higher content of metallic elements (Al, Cu, Zn, and Pb) was found (Table 24.2). Our results support the hypothesis that acid rains as well as other pollution sources increase the amount of heavy metals (especially Al) and flush out the bioelements (especially Ca and K) from soil and leaves. Based on these results, it was concluded that the changes of Rubisco activity and protein content could be used as a sensitive diagnostic parameter in ascertaining the negative effects of abiotic and biotic factors of the environment [142].



**TABLE 24.2**  
**Contents of the Metallic and Nonmetallic Elements**  
**in the Healthy and Damaged Oak Trees**

| Species               | Content of Elements (mg kg <sup>-1</sup> d.m.) |     |        |       |     |       |     |    |    |
|-----------------------|------------------------------------------------|-----|--------|-------|-----|-------|-----|----|----|
|                       | K                                              | Na  | Ca     | Mg    | S   | Al    | Cu  | Zn | Pb |
| Healthy samples       |                                                |     |        |       |     |       |     |    |    |
| <i>Q. cerris</i>      | 8,750                                          | 75  | 12,820 | 1,671 | 800 | 3,727 | 55  | 36 | 6  |
| <i>Q. robur</i>       | 8,470                                          | 100 | 24,120 | 2,023 | 810 | 1,090 | 78  | 62 | 3  |
| <i>Q. dalechampii</i> | 6,750                                          | 50  | 15,580 | 2,673 | 836 | 1,878 | 463 | 63 | 3  |
| Damaged samples       |                                                |     |        |       |     |       |     |    |    |
| <i>Q. cerris</i>      | 8,000                                          | 80  | 8,086  | 4,376 | 632 | 6,738 | 212 | 26 | 7  |
| <i>Q. robur</i>       | 6,620                                          | 100 | 8,288  | 851   | 824 | 1,697 | 68  | 23 | 2  |
| <i>Q. dalechampii</i> | 3,720                                          | 60  | 12,330 | 1,823 | 989 | 2,109 | 97  | 22 | 3  |

Source: Konečná, B. et al., *Photosynthetica*, 23, 566, 1989. With permission.

**24.5 WOODY PLANTS (WOODY TREES)**

In general, woody plants are used mainly as energetic plants (source of renewable energy) and in technological industry. Woodworking industry represents app. 40% of the total technically utilizable potential of biomass (wastes originated from mechanical processing of wood, filings, bark). Some of them (e.g., poplar, willow, black locust, ash, or alder) are not only fast growing species with high production potential but for their convenient biological features are also successfully used for the remediation of substrates contaminated by inorganic and organic pollutants. Mainly poplar has been shown to be an excellent species for phytoremediation purposes because it can be cultivated at high rates of growth and thus produce a large biomass. The use of plants producing large biomass for metal extraction from soil was proposed as an alternative to hyperaccumulators (these plants are mostly characterized by low biomass production) because high biomass production establishes the compensation of moderate heavy metal concentrations in their shoots. In general, the advantages of the genus *Populus* in phytoremediation are great number of species, fast growth up (3–5 m year<sup>-1</sup>), high transpiration rate (100 L day<sup>-1</sup>), and not being a part of food chains [143]. Fast growing trees have an extensive and massive root system penetrating deeply into the soil and ensuring efficient uptake of water containing the pollutants from the substrate. Moreover, these plants have perennial character, long life span, high transpiration rate, quick regeneration of the removed aboveground parts, and easy vegetative reproduction (see [144] for details). Poplars allow several cycles of decontamination, their leaves can be easily collected and the contaminated biomass is substantially reduced by incineration [145,146]. Although poplars are known to take up several inorganic pollutants including heavy metals, such as cadmium [147], mercury [148], and zinc [149], their heavy metal tolerance is limited [145]. It should be stressed that the precondition for the utilization of woody plants in phytoremediation technologies is their sufficient toxic metal tolerance. Therefore, the effects of metals (especially Cd, Hg, Pb, and Cu) on the structure and function of trees are still intensively studied. As cadmium belongs to the most dangerous environmental pollutants and has toxic and mutagenic effects on both, the plants and animals our attention was aimed on this toxic metal. In our earlier paper [150], we investigated the effect of Cd on root anatomy, growth, assimilation pigments, photosynthetic, and respiration rates of four willow species (*Salix viminalis* L., *S. alba* L., *S. purpurea* L., and *S. cinerea* L.) and two poplar species—*Populus × euroamerica* cv. Gigant and *Populus × euroamerica* cv. Robusta. It was found that the roots responded to Cd treatment more sensitively than the shoots. Cd-treatment suppressed rooting and root growth (length and biomass production)

as well as its development in all tested species. Root systems of *S. cinerea*, *S. alba*, and *Populus* cv. Robusta were more tolerant to Cd stress than the root system of other studied species. The analyses of Cd content in roots, cuttings, and shoots showed that Cd ions were accumulated mainly in the roots. Barceló and Poschenrieder [151] summarized the main morphological and structural effects of Cd on roots as follows: decrease of root elongation, root tip damage, collapsing of root hairs or decrease of their number, decrease of root biomass, and increase or decrease of lateral root formation. In another paper, the Cd effect on the above-mentioned willow and poplar species in response to different cultivation conditions was investigated [152]. The physiological and production parameters of the control plants were compared with plants grown at Cd treatment, and the plants rooted directly in  $\text{Cd}(\text{NO}_3)_2$  (direct treatment) were compared with plants firstly rooted in a Knop nutrient solution and transferred to  $\text{Cd}(\text{NO}_3)_2$  afterward (indirect treatment). It could be concluded that rooting in Knop nutrient solution when compared with direct cultivation in Cd had a positive impact on some production parameters of *S. alba* roots (root cumulative length, number, and biomass production) and some physiological characteristics of *S. alba* leaves (assimilation pigment and starch contents, net photosynthetic rate, and specific leaf mass). Roots and shoots of *P. robusta* rooted in Knop nutrient solution were more sensitive to the toxic effect of Cd than plants cultivated directly in Cd treatment. In general, cadmium negatively influenced root apices of both species in both experimental variants. On the other hand, mainly central cylinders of more distant root parts were not seriously influenced by Cd treatment. Cambial activity started and lateral root primordia were formed close to the root apex. Structural changes induced by Cd indicated a better adaptation of roots of directly Cd-treated plants of both species than of roots of indirectly Cd-treated plants. Cd enhanced values of specific leaf mass in both species caused xeromorphic character of leaves—increased stomata density but reduced stomata sizes. Assimilation pigment and starch contents, net photosynthetic rate, and specific leaf mass were positively influenced by indirect treatment. Indirect treatment lowered root Cd uptake in willow, Cd accumulation in cuttings of both species, and Cd accumulation in poplar shoot. Directly Cd-treated poplar roots exhibited an unusual defense activity of root apical meristem. Based on all obtained results, as well as above-mentioned physiological and production characteristics of fast growing woody plants, *S. alba* could be potentially used for phytoextraction of toxic metals from contaminated substrates. Similar results were published by Lunáčková et al. [153], but these authors additionally found that the Cd impact increased the root respiration rate of willow and poplar plants. Higher values of this physiological parameter was due to the fact that the toxic effect of Cd induced energy is required for an increased metal ions' uptake into the roots and for repairing mechanisms as a consequence of metabolism damages.

Nikolič et al. [154] observed symptoms of Cd toxicity in Cd-treated hybrid poplar plants ( $10\text{--}100\mu\text{mol dm}^{-3}$  Cd): stunted growth (plant height and biomass), decreased root length, and chlorosis of voting leaves. Stem and leaf growth was more affected than root growth. The decreased photosynthetic activity of treated plants may have been due to lowered chlorophyll synthesis. Cd concentration in roots was approximately 40 times higher than in leaves and stems of plants exposed to  $10^{-5}\text{M}$  Cd. Laureysens et al. [155] determined the total metal content in leaves, wood, and bark of 13 poplar clones. Cadmium, zinc, and aluminum were most efficiently taken up. The lowest concentration was found in wood; the highest concentrations were generally found in senescing leaves, making the removal and treatment of the fallen leaves necessary. Gu et al. [156] investigated the effect of  $\text{Cd}^{2+}$  ( $10$ ,  $50$ , and  $100\mu\text{mol dm}^{-3}$ ) on the growth of four poplar cultivars. Root growth was significantly inhibited at  $100\mu\text{M}$  Cd. Cd accumulation increased significantly with increasing Cd concentration and with time in all organs of the *Populus* cultivars. Cadmium concentrated mainly in the roots, and was higher than in the aerial parts. Cd concentrations were significantly higher in bark than in wood.

Jensen et al. [157] investigated the growth performance and heavy metal uptake by *Salix viminalis* in field and growth chamber trials and found that field uptakes were 2–10 times higher than growth chamber uptakes. Despite high concentrations of cadmium ( $\geq 80\text{ mg kg}^{-1}$ ) and zinc ( $\geq 3,000\text{ mg kg}^{-1}$ ) in leaves of willow grown on strongly polluted soil with up to  $18\text{ mg Cd kg}^{-1}$ ,  $1,400\text{ mg Cu kg}^{-1}$ ,

500 mg Pb kg<sup>-1</sup>, and 3,300 mg Zn kg<sup>-1</sup>, it is unsuited on strongly polluted soils because of poor growth. However, willow proved promising on moderately polluted soils (2.5 mg Cd kg<sup>-1</sup> and 400 mg Zn kg<sup>-1</sup>), where it extracted 0.13% of total Cd and 0.29% of the total Zn per year probably representing the most mobile fraction. Cu and Pb are strongly fixed in calcareous soils. Utmazian et al. [158] tested 20 different clones of willow and poplar species in hydroponic experiments for their metal resistance and accumulation properties. Metal concentration in hydroponics was 4.45 mmol dm<sup>-3</sup> Cd or 76.5 mmol dm<sup>-3</sup> Zn. The largest metal concentrations in leaves were detected in *Salix dasyclados* (315 mg Cd kg<sup>-1</sup> d.m.) and a *Salix smithiana* clone (3,180 mg Zn kg<sup>-1</sup> d.m.), but these species showed low metal tolerance. In spite of smaller Cd and Zn concentrations, the metal-tolerant clones *Salix matsudana*, *Salix fragilis*-1, and *Salix purpurea*-1 hold promise for phytoextraction as they produced large biomass and metal contents in leaves.

Vandecasteele et al. [159] investigated the growth and metal uptake of two willow clones (*Salix fragilis* “Belgisch Rood” and *Salix viminalis* “Aage”) cultivated in a greenhouse pot experiment using six sediment-derived soils with increasing field Cd levels (0.9–41.4 mg kg<sup>-1</sup>). Willow foliar Cd concentrations were strongly correlated with soil and soil water Cd concentrations. Both clones exhibited high accumulation levels of Cd and Zn in aboveground plant parts, making them suitable subjects for phytoextraction research. Interesting results were found by Mertens et al. [160] who cultivated several tree species on a mound constructed of dredged sediment slightly polluted with heavy metals. *Robinia pseudoacacia* and white poplar had the highest growth rates. Ash, maple, and alder had the highest survival rates (>90%) but showed stunted growth. Ash, alder, maple, and *R. pseudoacacia* contained normal concentrations of Cd, Cu, Pb, and Zn in their foliage. Consequently, these species reduce the risk of metal dispersal and are therefore suitable species for phytostabilization under the given conditions. White poplar accumulated high concentrations of Cd (8.0 mg kg<sup>-1</sup>) and Zn (465 mg kg<sup>-1</sup>) in its leaves and might therefore cause a risk of Cd and Zn input into the ecosystem because of autumn litter fall. This species is thus unsuitable for phytostabilization. Celik et al. [161] evaluated the leaves of *R. pseudoacacia* L. as biomonitors of heavy metal contamination in Denizli city, Turkey. Concentrations of Fe, Zn, Pb, Cu, Mn, and Cd were determined in washed and unwashed leaves and soils collected from a wide range of sites with different degrees of metal pollution (industry, urban roadside, and suburban) and from a rural (control) site. All the above-mentioned elements were found to be at high levels in samples collected at industrial sites, except for lead and copper that were found at high levels in samples collected from urban roadsides that associated with the road traffic. The strong correlation between the degree of contamination and concentrations in all plant leaves assessed display that the leaves of *R. pseudoacacia* reflect the environmental changes accurately, and that they seem as an effective biomonitor (bioindicator) of environmental quality in areas subjected to industrial and traffic pollutions.

## 24.6 METAL TOXICITY-INDUCED ALTERATIONS IN CROPS

From a global perspective, it can be stated that crops are predominantly used as food and fodder. Only in advanced and highly developed countries are these plants also used as technical plants (e.g., for alternative source of energy or environment protection). Therefore, the ethical aspect is emphasized if the crops (e.g., maize, cereals, potatoes, rapeseed, and sunflower) could be used exclusively for alimentary purposes or also as an alternative energy source. Moreover, the effect of the toxic substances including heavy metals on physiological and production characteristics of the crops is in general extraordinarily important. Considering all the above-mentioned aspects of the crops, this would require a separate chapter in this book. However, we want to present a brief choice related to the effect of metals on cytology, anatomy, physiology, and production of these important group of plants.

Toxic metals are known as mitotic inhibitors that finally cause the reduction of root growth. Jiang et al. [162] observed the toxic effect of CuSO<sub>4</sub> (10<sup>-4</sup> to 10<sup>-1</sup> mol dm<sup>-3</sup>) on chromosomal morphology.

Doncheva [163] found that copper interrupts the progression of nuclei at the crucial G1/S transition point of the cell cycle, when it prevents their entry into mitosis. The decreased root growth could be due to the effect of copper on the root–meristem cell proliferation. In *Hordeum vulgare* and *Setaria italica* plants Yadav and Srivastava [164] confirmed the inhibitory effect of  $\text{Cd}^{2+}$  ions on the mitotic and active mitotic indices whereby  $\text{Cd}^{2+}$  ions induced various types of mitotic anomalies.

Ivanov [165] investigated the inhibition of maize root growth by metal ions and found that the inhibitory efficiency decreased in the following order:  $\text{Cu} \approx \text{Tl} > \text{Ag} > \text{Cd} > \text{Hg} > \text{Co} > \text{Zn} > \text{Pb}$  (with concentrations calculated as  $\text{g dm}^{-3}$ ) and  $\text{Tl}^{3+} > \text{Cu}^{2+} \gg \text{Ag}^+ > \text{Hg}^{2+} \approx \text{Cd}^{2+} > \text{Zn}^{2+} \approx \text{Pb}^{2+} \approx \text{Co}^{2+}$  (for molar concentrations). Metal affinity for SH-groups of biological compounds was closely correlated ( $r = 0.9$ ) with the molar concentration that inhibited primary root growth by 50%. A similar sequence of the efficiency of phytotoxic effects of metals reflected in root growth inhibition ( $\text{Cu}^{2+} > \text{Hg}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+}$ ) was also observed in tests with *Brassica napus* plants [166]. Sagardoy et al. [167] ascertained that the leaves of hydroponically cultivated sugar beet (*Beta vulgaris* L.) plants treated with 50 and 100  $\mu\text{M}$  zinc sulfate developed symptoms of Fe deficiency, including decreases in Fe, chlorophyll, and carotenoid concentrations; increases in carotenoid/chlorophyll and chlorophyll *a/b* ratios; and depoxidation of violaxanthin cycle pigments. Sunakar and Sumita [168] published that although lead ion ( $\text{Pb}^{2+}$ ) induces thylakoid-membrane lipid peroxidation, it prevents the loss of pigments (chlorophylls and carotenoids) and loss of proteins. The cation-induced retardation in pigment and protein loss is explained in terms of metal ion binding to lipid protein complex through carboxylic acid group of lipids and sulfhydryl group of protein. Chu et al. [169] using pot-culture experiments examined the individual and combined effects of Cu and Cd pollutants on *Trifolium repens* L. seedlings and observed that the contents of their leaf pigments decreased. Chlorophyll *a* was more sensitive than chlorophyll *b* to Cu and Cd pollutants, and chlorophyll *b* was more sensitive than carotenoid. It was also shown that the active oxygen metabolism of *T. repens* seedlings was destroyed by high amounts of Cu and Cd.

Souza et al. [170] using scanning electron microscopy for the investigation of 8 day old maize plants exposed to Cd and Zn observed changes in the leaf surface, particularly in the guard cells of the stomata. The ultrastructural analyses of the parenchyma mesophyll cells showed extensive chloroplast disorganization, mainly affecting the thylakoid membranes and grana.

Hydroponically cultivated seedlings of the soybean (*Glycine max* (L.) Merr.) exposed to  $\text{PbCl}_2$  (0, 10, 20, and 40  $\text{mg dm}^{-3}$ ) were characterized by Pb-induced changes in the leaf epidermis structure involving a reduction in the cell size, more abundant wax coating, and an increase in the number of stomata and trichomes per unit leaf area with simultaneous reduction in the size of the guard cells [171]. Burst stroma of the thylakoid system and the cracked chloroplast envelopes were also observed. The importance of the increase in the number of stomata and trichomes for plants under the metal stress was examined.

Kastori et al. [114] found that excess concentrations of lead (Pb), cadmium (Cd), copper (Cu), and zinc (Zn) significantly decreased transpiration and relative water content in hydroponically cultivated young sunflower (*Helianthus annuus* L.) plants. On the other hand, the number of stomata per unit leaf area increased while the size of the stomata decreased. Thus, excess concentrations of the heavy metals significantly affected plant water status, causing water deficit and subsequent changes in the plants. Cd exerted the most intensive effect on the plants, less intensive effects were found for Cu and Zn, at least harmful by Pb.

Llamas et al. [172] investigated  $\text{Cd}^{2+}$  effects on transmembrane electrical potential difference, respiration, and membrane permeability of rice (*Oryza sativa* L.) roots and found that upon addition of 0.1 or 1  $\text{mmol dm}^{-3}$   $\text{Cd}^{2+}$  to the experimental solution, root cell membranes depolarized in few minutes, reaching very low  $E_m$  values. This effect was transient and the initial membrane potential recovered totally within 6–8 h. Only the highest concentration used had an inhibitory effect on root respiration. Significant respiratory inhibition appeared after 2 h of exposure to  $\text{Cd}^{2+}$  and lasted for at least 4 h. In turn, membrane permeability increased in the presence of  $\text{Cd}^{2+}$  for at least 8 h, inducing  $\text{K}^+$  efflux from the roots. According to Burzynski and Buczek [173], application of 10  $\mu\text{mol dm}^{-3}$  Cd, Cu, and Ni, and 50  $\mu\text{mol dm}^{-3}$  Pb did not change the intensity of root respiration of cucumber

seedlings. Moreover, the authors found that the respiration of cucumber roots during one hour of tissue incubation with heavy metals in concentrations inhibiting  $\text{NO}_3^-$  absorption was similar as in the control and thus the decrease in nitrate uptake by seedlings exposed to Cd, Cu, Ni, or Pb was not due to the influence of these metals on the respiratory processes.

Metal toxicity-induced alterations in crop photosynthesis (different organization levels) were published by many authors, see, e.g., [174–178] for details.

It should be stressed that the final consequence of the above-mentioned negative effects of toxic metals on crops is manifested in an undesirable decrease of crop yield, what can seriously affect the whole human population [179–181].

## 24.7 RESPONSE OF MEDICINAL PLANTS TO METAL PRESENCE

It was recognized that out of the 350,000 vascular plant species identified so far, about 35,000 (the estimates vary) species have at one time or the other been used by some people or cultures for medicinal purpose [182]. According to research carried out by the World Wildlife Fund (WWF), up to 90% of species of medicinal and aromatic plants (MAPs) traded in Europe are still harvested from the wild, and a rapid growth in the market is now resulting in the over-exploitation of wild stocks of some species [183]. Totally, from about 2,000 MAPs traded in Europe, 1,200–1,300 are native to the continent with only 130–140 species predominantly derived from cultivated stock. Wild harvesting of MAPs in Europe is still prominent in many former Eastern Bloc countries including Slovakia where the climate, soil, and low levels of pollution in these countries are some of the best in middle Europe for the cultivation of medicinal plants. Details concerning the above-mentioned topic can be found in the project of Interactive European Network for Industrial Crops and their Applications, IENICA INFORRM Project [184]. Medicinal plants could be regarded as potential plant factories for new natural drugs. Hence, it could be stressed that it is necessary to check and monitor the herbs for the content of harmful substances including toxic metals (see [185] for details). Additionally, medicinal plants have a great potential for their exploitation in modern phytotechnologies, such as phytoremediation and phytofortification [186].

At present, it can be stated that the anthropogenic activity and its effects on the environment showed that medicinal plants have also responded to the changing environmental conditions. Some medicinal plants produce specific secondary metabolites that can detoxify some of the toxic metals. *Hypericum perforatum* and *Matricaria recutita* belong to cadmium hyperaccumulators because they accumulate in their shoots over  $100\mu\text{g g}^{-1}$  d.m. This property can be used in the future in phytoremediation technologies. Moreover, the rest of the biomass of medicinal plants after drug isolation can be utilized as organic fertilizers and pesticide preparations. On the other hand, in the last years, the practical use of alternative medicine in healing processes showed a continually increasing tendency. Several species of medicinal plants can be used as supplementary nutrition due to their ability to accumulate some essential nutrition elements (e.g., Se, Zn, and Fe) in the edible parts of these plants. Such fortification of plants with essential nutrients (phytofortification) in an easily assimilated form can help to feed the rapidly increasing world population and improve human health through a balanced mineral nutrition. In general, data related to toxic metal contents (e.g., Cd) in pharmaceutically utilized parts of the medicinal plants are also considered from the aspect of “food safety”(see [186] for details).

In our experiments, we focused on three medicinal species: *Hypericum perforatum* L., *Matricaria recutita* L., and *Salvia officinalis* L. that are in general the most frequent medicinal plants used in phytotherapy. Some of the found and important results are presented in the following text.

*Hypericum perforatum* is a plant that has been used as a medicinal herb since ancient time. Major constituents of this plant extracts include several classes of compounds exemplified by flavonols, flavonol glycosides, biflavones, naphthodianthrone, phloroglucinols, tannins, coumarins, essential oils, xanthophylls, and others [187]. The content of naphthodianthrone derivatives hypericin and pseudohypericin is approximately 0.05%–0.15%, that of flavonoid quercetin and biflavone

biapigenin is 0.3% and 0.26%, respectively. From the other secondary metabolites, the highest content belongs to phloroglucinole derivatives hyperforin and adhyperforin (up to 4%) [188]. These compounds are very important for the medicinal plants to preserve them against environmental stress. Thus, we studied the tolerance of *H. perforatum* to the toxic effect of copper and cadmium with respect to metal accumulation in individual plant organs [189]. The 6 week (Cu experiment) or 5 month old plants (Cd experiment) were exposed in hydroponics for 7 days to the following metal concentrations: 15, 30, 60, 90, and 120  $\mu\text{mol dm}^{-3}$   $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  and 12  $\mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ . Thereafter, the length and dry mass of shoots and roots were measured and the metal content in the plant organs determined. It was confirmed that as the most sensitive parameter to Cu treatment was found to be the root dry mass. The length of shoot as well as shoot dry mass was not significantly affected. Lower values of root dry mass could be explained with a significant reduction of lateral roots and root hairs by Cu treatment. The roots of *H. perforatum* accumulated markedly higher concentrations of Cu than the shoots. The metal accumulated in both plant organs showed an increase with increasing metal concentration. Bioaccumulation factors (BAF), that is, quotients obtained by dividing the concentration of the metal in dry mass of individual plant tissues (root and shoot, respectively) by its concentration in the external exposure medium, were also calculated (Table 24.3). Taking into account the actual dry mass of individual plant organs (root and shoot), the Cu concentration in the shoot was within 20% of the investigated concentration range of total metal content uptake by the whole plant (Table 24.3,  $P_{\text{shoot}}$ ). With respect to relatively high Cd content in the shoot dry mass (1,087  $\mu\text{g g}^{-1}$ ), *H. perforatum* could be classified as a Cd hyperaccumulator. In this chapter, the possible contribution of the formation of metal complexes with the secondary metabolites of *H. perforatum* to the plant metal tolerance has been discussed first. Later, we stated [186] that for

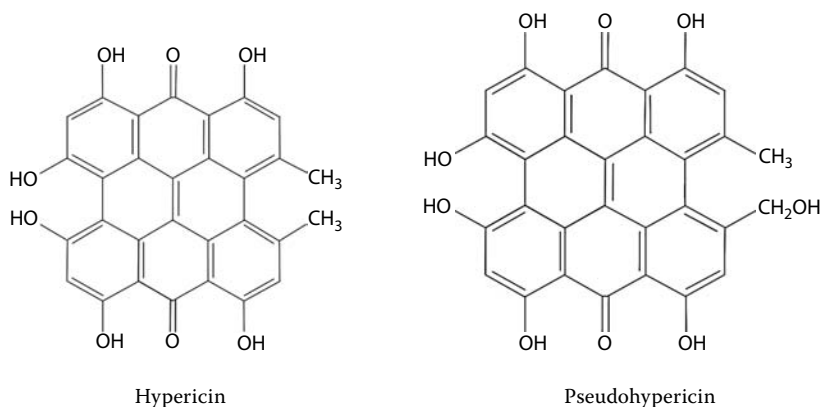
**TABLE 24.3**  
**Metal Content in Dry Mass of *H. perforatum* Plant Organs ( $\mu\text{g g}^{-1}$  d.m.)**

| Metal | Plant Organ | Ext. Metal Conc.<br>( $\mu\text{mol dm}^{-3}$ ) | Metal Content in<br>Dry Mass ( $\mu\text{g g}^{-1}$ ) | BAF   | S/R Ratio $\times 10^2$ | $P_{\text{shoot}}$ |
|-------|-------------|-------------------------------------------------|-------------------------------------------------------|-------|-------------------------|--------------------|
| Cu    | Root        | 0                                               | 56.6                                                  | —     | —                       | 0.173              |
|       | Shoot       | 0                                               | 13.3                                                  | —     | —                       |                    |
|       | Root        | 15                                              | 1616                                                  | 1695  | 1.67                    |                    |
|       | Shoot       | 15                                              | 27.0                                                  | 28.33 | —                       |                    |
|       | Root        | 30                                              | 3698                                                  | 1940  | 1.68                    | 0.217              |
|       | Shoot       | 30                                              | 62.1                                                  | 32.57 | —                       |                    |
|       | Root        | 60                                              | 5874                                                  | 970   | 3.15                    | 0.232              |
|       | Shoot       | 60                                              | 116.5                                                 | 30.55 | —                       |                    |
|       | Root        | 90                                              | 7856                                                  | 1376  | 1.80                    | 0.228              |
|       | Shoot       | 90                                              | 141.8                                                 | 24.79 | —                       |                    |
|       | Root        | 120                                             | 6767                                                  | 887   | 2.29                    | 0.209              |
|       | Shoot       | 120                                             | 154.3                                                 | 20.33 | —                       |                    |
| Cd    | Root        | 0                                               | <1                                                    | —     | —                       | —                  |
|       | Shoot       | 0                                               | <1                                                    | —     | —                       |                    |
|       | Root        | 12                                              | 7262                                                  | 5382  | 15.0                    | 0.240              |
|       | Shoot       | 12                                              | 1087                                                  | 806   | —                       |                    |

Source: Král'ová, K. and Masarovičová, E., in *Macro and Trace Elements*, Anke, M. et al. (eds.), Mengen- und Spurenelemente, 22. Workshop, Friedrich Schiller Universität, Jena, Germany, September 24–25, 2004, pp. 411–416, 2004. With permission.

Note: Duration of cultivation in hydroponic solution: 7 days; BAF, bioaccumulation factor; S/R, metal content in dry mass of the shoot: metal content in dry mass of the root;  $P_{\text{shoot}}$ , portion of metal in the shoot to the total accumulated metal content in the whole plant.

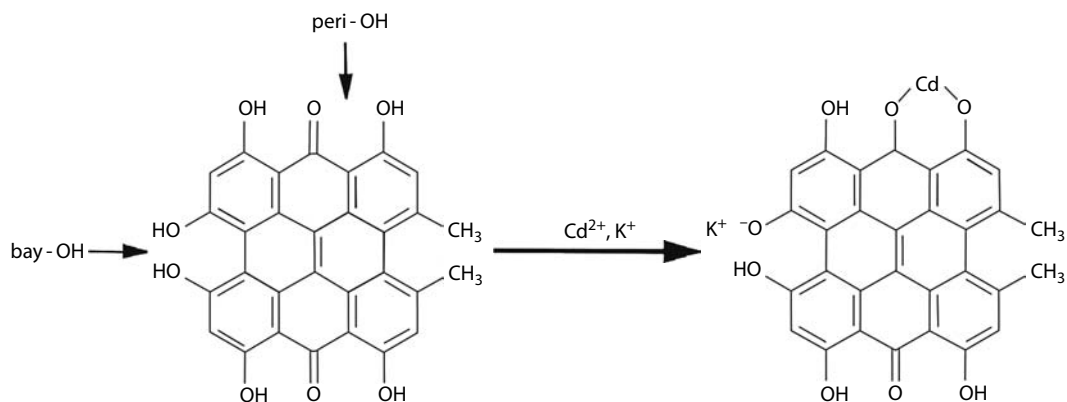
plants producing specific secondary metabolites (medicinal plants), further additive mechanism of tolerance arose. This additive mechanism is connected with chelation of metal ions by some specific secondary metabolites, such as hypericin and pseudohypericin produced by *H. perforatum*. The toxic ionic form of metal is thus changed into nontoxic metal chelate. This assumption is based on the findings of Falk and Schmitzberger [190] and Falk and Mayr [191] who stated that the pronounced acidity of the *bay*-region hydroxyl groups of hypericin makes salt formation a definite possibility: hypericin is present in the plant material mainly as its potassium salt. In addition, in structurally similar *bay*-hydroxylated fringelites salt formation with divalent ions, such as  $\text{Ca}^{2+}$ , yields polymeric systems, which, because of their extreme insolubility, are highly stable in fossils.



The *peri*-hydroxyl groups situated in the neighborhood of the carbonyl groups display the best prerequisites to form chelates with transition metal ions. Such coordination complexes could be characterized in the case of fringelite D and  $\text{Zn}^{2+}$  [191].

Afterward, Palivan et al. [192] investigated the formation of copper complexes with hypericin in solutions using EPR spectroscopy and found that hypericin forms a four-coordinated copper species where the solvent participates in the coordination sphere of the metal. An excess of metal was not compulsory for the formation of the hypericin–copper complex; however, a higher aggregate (chain structure) could not be ruled out.

Taking into account the above-mentioned results concerning complex formation between hypericin and copper, it could be assumed that this secondary metabolite of *H. perforatum* as well as the structurally similar pseudohypericin will form similar complexes with further transition metal ions. Due to such interaction, the concentration of free metal ions will decrease and their toxic effect will be diminished. Thus, the formation of complexes between heavy metals and the above-mentioned secondary metabolites could be regarded as a further mechanism contributing to the enhanced tolerance of *H. perforatum* against such divalent metals as copper and cadmium.



The above-described and illustrated chelation of cadmium ions with hypericin could contribute to the enhanced tolerance of *Hypericum perforatum* plants to cadmium stress and to their cadmium hyperaccumulating ability. Classification of this medicinal plant species as a Cd hyperaccumulator firstly was confirmed by Marquard and Schneider [193] and consequently also in our research [194,195].

According to Murch et al. [196], metal contamination can change the chemical composition of *Hypericum perforatum*, thereby seriously impacting the quality, safety, and efficacy of natural plant products. In the presence of 25 and 50 mM Ni plants lost completely the capacity to produce or accumulate hyperforin and demonstrated a 15–20-fold decrease in the concentration of pseudohypericin and hypericin. Similarly, total alkaloid was also found to be decreased in the roots of *Catharanthus roseus* (L.) plants treated with CdCl<sub>2</sub> [197].

Several authors have suggested that *Matricaria recutita* species tolerate Cd concentrations corresponding to the middle-strong contaminated soils [198,199], and high Cd concentration in their shoots assigns this medicinal plant as a Cd hyperaccumulator. However, the existence of the above-discussed additive mechanism of tolerance due to chelate formation between some chamomile secondary metabolites and cadmium has to be experimentally confirmed.

In our paper [195], the effect of cadmium (12 μM Cd(NO<sub>3</sub>)<sub>2</sub>; pH = 5.5) on growth, plant biomass (root and shoot), and root dark respiration rate of *H. perforatum* (cultivated hydroponically) as well as cadmium accumulation in all plant organs was investigated. The highest Cd concentration was found in the root (1,792 μg g<sup>-1</sup> d.w.) that has eight times higher concentration than in the stem and six times higher than that in the leaves. Based on these results it could be concluded that Cd supported the release (permeability) of membranes in both roots and shoots. Consequently, the above-mentioned metal ions were transported into the leaves where their higher content was estimated. The effect of cadmium treatment on the content of iron, manganese, and copper in individual plant organs has been also determined (Table 24.4). Cd administration did not affect the growth and dry biomass of the shoot and root, and the root:shoot ratio. However, the root dark respiration rate of the Cd-treated plants was faster than those of control plants (Table 24.5) [195].

It has already been mentioned that *Matricaria recutita* L. belongs to the most favored medicinal plant not only in Slovakia but also all over the world [186]. From a historical perspective, the native

**TABLE 24.4**  
**Metal Contents in Control and Cadmium-Treated**  
***H. perforatum* Plant Organs**

| Plant Organs | Accumulated Metal (μg g <sup>-1</sup> d.m.) |              |              |              |
|--------------|---------------------------------------------|--------------|--------------|--------------|
|              | Cd                                          | Mn           | Fe           | Cu           |
| Root—control | <1                                          | 15.6         | 344.8        | 65.8         |
| Root—treated | 1792 (>1792)                                | 9.2 (59.0)   | 263.7 (76.5) | 49.8 (75.7)  |
| Stem—control | <1                                          | 1.4          | 55.4         | 18.3         |
| Stem—treated | 220 (>220)                                  | 0.6 (42.9)   | 18.7 (33.8)  | 1.1 (6.0)    |
| Leaf—control | <1                                          | 9.7          | 43.5         | 7.0          |
| Leaf—treated | 290 (>290)                                  | 13.3 (137.1) | 63.8 (146.7) | 10.0 (142.9) |

Source: Král'ová, K. and Masarovičová, E., in *Macro and Trace Elements*, Anke, M. et al. (eds.), Mengen- und Spurenelemente, 22. Workshop, Friedrich Schiller Universität, Jena, Germany, September 24–25, 2004, pp. 411–416, 2004. With permission.

Note: The values in the brackets presented for individual metal contents in cadmium-treated plant organs correspond to the percentage of corresponding control value.



**TABLE 24.5**  
**Dark Respiration Rate of the Root, Shoot,**  
**and Root Biomass, Root:Shoot Ratio, and Cd**  
**Concentration in the Plant Organs of *Hypericum***  
***perforatum* Cultivated under Cd Application**

| Parameters                                                                                     | Control           | Cd Treatment                  |
|------------------------------------------------------------------------------------------------|-------------------|-------------------------------|
| Dark respiration rate of the root<br>( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ d.w. s}^{-1}$ ) | $1.372 \pm 0.079$ | $1.885 \pm 0.156^*$           |
| Root biomass (g d.w.)                                                                          | $0.130 \pm 0.018$ | $0.104 \pm 0.005^{\text{ns}}$ |
| Shoot biomass (g d.w.)                                                                         | $0.257 \pm 0.045$ | $0.176 \pm 0.026^{\text{ns}}$ |
| Total biomass (g d.w.)                                                                         | $0.387 \pm 0.060$ | $0.280 \pm 0.027^{\text{ns}}$ |
| Root: shoot ratio                                                                              | $0.526 \pm 0.074$ | $0.623 \pm 0.077^{\text{ns}}$ |

Source: Král'ová, K. et al., *Chem. Inz. Ekol.*, 7, 1200, 2000. With permission.

Note: Significant differences between control and Cd treated plants are indicated by \* ( $p = 0.05$ ) or by <sup>ns</sup> meaning no significant difference.

areas of the chamomile species were Asia, Northern Africa, and Southern and Eastern Europe. The notions about chamomile healing power came from antique scientific works of Hippocrates, Pliny, Dioscorides, and Galen into old herbals and current phytomedicine. Chamomile was already introduced in the register of synantropic plants of vegetation of settlements of Bratislava and Trnava from 1774 and 1791, respectively [200]. In mythology, chamomile belongs to the nine holy herbs which Odin, the main god of North-European German folks, donated to mortals for the improvement of their life. Nowadays, chamomile (*Matricaria recutita* L., earlier also synonyms *Chamomilla recutita* (L.) Rausch. or *Matricaria chamomilla* L.) is the most favored and most frequently used medicinal plant over the world. In the former Czechoslovakia, *M. recutita* represented the base of more than 32 official mass-produced phytotherapeutic preparations [201].

*M. recutita* produces a variety of volatile secondary metabolites, e.g., chamomillol, gossanol, cubenol,  $\alpha$ -cadinol, chamazulene,  $\beta$ -farnesene, (–)- $\alpha$ -bisabolol, (–)- $\alpha$ -bisabololoxide A, (–)- $\alpha$ -bisabololoxide B, 1-azulenethanol acetate and (–)- $\alpha$ -bisabolol acetate [202], herniarin [203], etc. Schlicher in 1973 [204] identified four main chemical types of intraspecific variability of chamomile based on the composition of its essential oil. For the chamomile varieties cultivated in Slovakia, it is characteristic that the content of important secondary metabolites decreases in the following order:  $\alpha$ -bisabolole >  $\alpha$ -bisabolole oxide B >  $\alpha$ -bisabolole oxide A, that is, these cultivars belong to the chemical type A of *M. recutita* (see [205] for details).

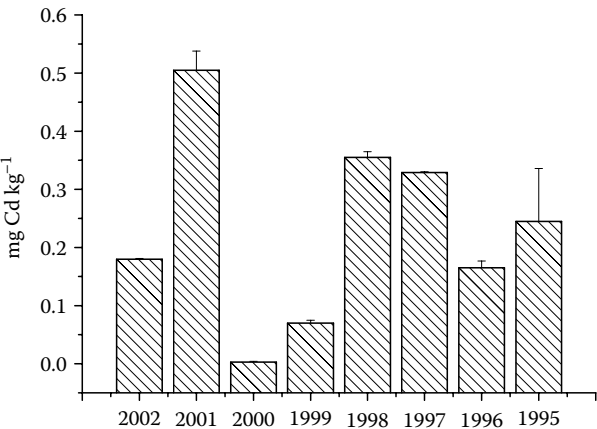
Traditionally, in Eastern Slovakia, large regions are used for commercial chamomile cultivation. As chamomile species are long-term cultivated in field conditions, it is important to know how many of the Cd is taken up from the soil, transported, and accumulated in individual parts of plants. Therefore, the Cd content in the pharmaceutically important plant part—anthodium—was also estimated [205]. The values of Cd concentrations in the soil (determined in 2M HNO<sub>3</sub> leachate) and in chamomile anthodium as well as corresponding BAF related to some localities in Slovakia in which field cultivation of chamomile occurs (for the period 1999–2001) are shown in Table 24.6. The presented BAF are quotients obtained by dividing the concentration of Cd in the dry mass of anthodium by its concentration in the soil. The Cd fluctuation in chamomile anthodium (1995–2002) related to plants cultivated in different localities of Eastern Slovakia is presented in Figure 24.9.

We also evaluated the relationship between the Cd content in chamomile anthodium dry mass and mean hydrothermic coefficient of Seljaninov (HC) as an integrated index of hydrothermic parameters. This coefficient could be calculated according to the following formula:

**TABLE 24.6**  
**Cadmium Concentrations in Soil (Determined in 2 M HNO<sub>3</sub> Leachate) and in Chamomile Anthodium Dry Mass as well as Corresponding BAF Related to Some Localities in Slovakia in Which Field Cultivation of Chamomile Occurs**

| Locality            | Cd in Soil (mg kg <sup>-1</sup> ) | Cd in Anthodia (mg kg <sup>-1</sup> ) | BAF   |
|---------------------|-----------------------------------|---------------------------------------|-------|
| Streda nad Bodrogom | 0.111 ± 0.042                     | 0.168 ± 0.078                         | 1.514 |
| Košice              | 0.334 ± 0.050                     | 0.078 ± 0.022                         | 0.234 |
| Michalovce          | 0.335 ± 0.054                     | 0.179 ± 0.112                         | 0.534 |
| Nová Lubovňa        | 0.222 ± 0.025                     | 0.150 ± 0.021                         | 0.676 |

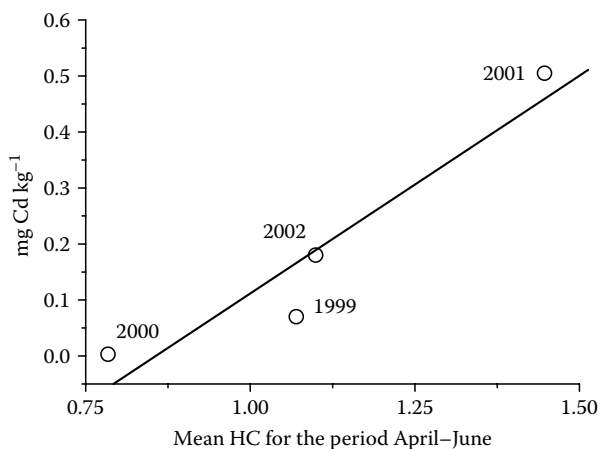
Source: Šalamon, I. et al., *Acta Hort. (ISHS)*, 749, 217, 2007. With permission.  
Note: The data are related to the period 1999–2001.



**FIGURE 24.9** Cadmium content in anthodium of chamomile plants cultivated and collected in different localities of Eastern Slovakia in the period 1995–2002. (From Šalamon, I. et al., *Acta Hort. (ISHS)*, 749, 217, 2007. With permission.)

$HC = \Sigma R / (0.1 \times TS_m)$ , where  $\Sigma R$  is the total amount of precipitations and  $TS_m$  is the thermal sum of the mean daily temperatures exceeding 10°C in the investigated period. The dependence of Cd content in the chamomile anthodium dry mass on the mean hydrothermic coefficient for the period April–June evaluated from the data measured in the meteorological stations Streda nad Bodrogom and Michalovce in the period 1995–2002 is presented in Figure 24.10. The correlation between the accumulated Cd and HC value supported enormous significance of the actual climatic relations on the metal uptake and accumulation.

Marquard and Schneider [193] were the first to confirm that chamomile plants had the potential to accumulate high levels of cadmium from the soil. In our paper [206], two tetraploid cultivars of *Matricaria recutita* L. (cv. Goral and cv. Lutea) were investigated in response to Cd application. Treated plants were cultivated in Hoagland solution with the following Cd concentrations: 3, 6, 12, 24, and 60 μmol dm<sup>-3</sup> Cd(NO<sub>3</sub>)<sub>2</sub>. The plants growing in Hoagland solution without Cd served as control. Primary root length, root increment (root length after treatment – root length before treatment) was calculated [(root increment of Cd-treated plants/root increment of control plants) × 100] in %, root and shoot dry mass, as well as Cd content in plant organs were determined after 7 days of treatment. In other experiments, the older plants were grown in the greenhouse conditions in the soil for 7 weeks after germination. Plants used for photosynthetic and respiration measurements as well



**FIGURE 24.10** Dependence of Cd content in chamomile anthodium dry mass on the mean HC for the period April–June evaluated from the data measured in the meteorological stations Streda nad Bodrogom and Michalovce. (From Šalamon, I. et al., *Acta Hort. (ISHS)*, 749, 217, 2007. With permission.)

as for the analysis of assimilation pigment concentration were grown under greenhouse conditions for 9 weeks after germination. Then their roots were washed, transferred to hydroponic Hoagland solutions (control), and Hoagland solution with  $12\mu\text{M Cd}(\text{NO}_3)_2$  and placed in the growth chamber for 10 days. The concentration gradient that was used in our experiments reflects the Cd content in the soils from non-contaminated to highly contaminated sites [207]. For the estimation on Cd toxicity of roots, we used primary root length and root increment, which are considered a reliable parameter for heavy metal tolerance [208]. At the beginning of Cd-treatment, nonsignificant variance in root length was observed. Significant inhibition of root growth was observed in both the chamomile cultivars after Cd-treatment (Table 24.7). We did not find any differences in the Cd accumulation in the roots of different cultivars, but cv Lutea accumulated a slightly higher amount of Cd in the shoot. No differences between the cultivars were recorded after Cd-treatment in growth parameters (Table 24.7). In the root test, we observed fragility, browning, and twisting of roots. In the shoots, leaf roll, chlorosis, and leaf growth inhibition occurred. During the root test chamomile plants cv. Goral formed the anthodia in all the concentrations except that of the control, despite the fact that the plants were only 3 weeks old. According to our observation, the plants started blossoming when they are 8–12 weeks old; however, Cd treatment resulted in reduced size of flowers. In the Cd hyperaccumulator *Arabidopsis halleri*, 4–5 weeks earlier blossoming under Cd administration was recorded [209]. Cd concentration  $12\mu\text{mol dm}^{-3}$  in the hydroponic solution (used in our other experiment, Table 24.8) represents strongly contaminated soil [207], however, the Cd effect on the plant was stronger in comparison to the soil, because Cd is not bound to the soil particles and so all the ions are available for plant uptake. cv. Lutea seemed to be more sensitive to Cd treatment, e.g., it exhibited greater leaf chlorosis. The measurements confirmed a higher inhibition of photosynthesis in cv. Lutea, although they accumulated less Cd than cv. Goral. Similar decrease of shoot dry weight in both cultivars was also detected (Table 24.8). Decrease of net photosynthetic rate could be due to structural and functional disorders in many different levels. Shoot and root respiration rates were not changed significantly in both chamomile cultivars (Table 24.8). We confirmed that chamomile belongs to the group of Cd accumulator species. If we take into account the high content of Cd in chamomile shoot (over  $300\mu\text{g g}^{-1}$  at  $12\mu\text{mol dm}^{-3}$  Cd in solution), only a small extent of damages occurred in Cd treated plants. Therefore, this medicinal plant species exhibited high tolerance to Cd treatment. This was also confirmed by Masarovičová et al. [199] through the effect of cadmium and zinc separately ( $10\mu\text{mol dm}^{-3}$  for Cd and  $50\mu\text{mol dm}^{-3}$  for Zn), the combined application of these ions on physiological processes (photosynthetic rate, dark respiration rates of leaves and roots, and

TABLE 24.7

Concentration of Cd in Solution in Relation to Cd Accumulation, and Cd Accumulation in Relation to Growth Parameter (Root Length, Shoot, and Root Weight)

| Species and Cultivar                 | Cd in Solution ( $\mu\text{M}$ ) | Root Cd Accumulation ( $\mu\text{g g}^{-1}$ d.w.) <sup>a</sup> | Shoot Cd Accumulation ( $\mu\text{g g}^{-1}$ d.w.) <sup>a</sup> | Root Weight (mg d.w.)                              | Shoot Weight (mg d.w.)                             | Primary Root Length (cm)                           | Root Increment (%) |
|--------------------------------------|----------------------------------|----------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------|----------------------------------------------------|----------------------------------------------------|--------------------|
| <i>Matricaria recutita</i> cv. Goral | Control                          | 12.2                                                           | 6.0                                                             | $3.92 \pm 0.29$                                    | $14.21 \pm 1.55$                                   | $8.73 \pm 0.44$                                    | 100                |
|                                      | 3                                | 103.8                                                          | 32.1                                                            | $3.01 \pm 0.28$                                    | $10.62 \pm 1.15$                                   | $7.83 \pm 0.36$                                    | 78                 |
|                                      | 6                                | 236.0                                                          | 41.8                                                            | $3.16 \pm 0.41$                                    | $9.52 \pm 1.21$                                    | $8.01 \pm 0.55$                                    | 91                 |
|                                      | 12                               | 323.0                                                          | 50.2                                                            | $2.87 \pm 0.53$                                    | $9.76 \pm 1.12$                                    | $7.60 \pm 0.55$                                    | 71                 |
|                                      | 24                               | 412.5                                                          | 66.2                                                            | $2.08 \pm 0.13$                                    | $8.89 \pm 1.05$                                    | $7.14 \pm 0.54$                                    | 65                 |
|                                      | 60                               | 695.0                                                          | 117.3                                                           | $1.53 \pm 0.12$                                    | $5.99 \pm 0.59$                                    | $5.60 \pm 0.21$                                    | 39                 |
| Regression equation                  |                                  | $y = 370.8x - 42.4$<br>$R^2 = 0.95$<br>$p < 0.001$             | $y = 56.4x + 0.6$<br>$R^2 = 0.91$<br>$p = 0.002$                | $y = -0.003x + 3.7$<br>$R^2 = 0.85$<br>$p = 0.009$ | $y = -0.067x + 13.2$<br>$R^2 = 0.84$<br>$p = 0.01$ | $y = -0.004x + 8.7$<br>$R^2 = 0.93$<br>$p = 0.002$ |                    |
| <i>Matricaria recutita</i> cv. Lutea | Control                          | 10.6                                                           | 5.5                                                             | $6.29 \pm 0.76$                                    | $13.29 \pm 1.33$                                   | $11.93 \pm 0.35$                                   | 100                |
|                                      | 3                                | 139.3                                                          | 51.8                                                            | $4.81 \pm 0.62$                                    | $10.37 \pm 1.31$                                   | $10.62 \pm 0.42$                                   | 77                 |
|                                      | 6                                | 226.0                                                          | 68.0                                                            | $3.73 \pm 0.57$                                    | $8.55 \pm 1.09$                                    | $9.37 \pm 0.36$                                    | 72                 |
|                                      | 12                               | 313.0                                                          | 75.4                                                            | $2.51 \pm 0.17$                                    | $8.36 \pm 0.99$                                    | $7.80 \pm 0.41$                                    | 42                 |
|                                      | 24                               | 381.2                                                          | 91.2                                                            | $2.92 \pm 0.53$                                    | $8.42 \pm 1.23$                                    | $7.40 \pm 0.31$                                    | 42                 |
|                                      | 60                               | 702.0                                                          | 140.5                                                           | $3.62 \pm 0.78$                                    | $9.24 \pm 0.80$                                    | $7.37 \pm 0.30$                                    | 38                 |
| Regression equation                  |                                  | $y = 359.6x - 33.9$<br>$R^2 = 0.95$<br>$p < 0.001$             | $y = 68.2x + 9.6$<br>$R^2 = 0.95$<br>$p < 0.001$                | $y = -0.004x + 5.1$<br>$R^2 = 0.40$<br>$p = 0.18$  | $y = -0.031x + 11.9$<br>$R^2 = 0.52$<br>$p = 0.08$ | $y = -0.007x + 11.1$<br>$R^2 = 0.74$<br>$p = 0.03$ |                    |
| ANCOVA                               |                                  | Slope $p = 0.867$<br>$y$ -inter $P = 0.962$                    | Slope $p = 0.962$<br>$y$ -inter $P = 0.025$                     | Slope $p = 0.853$<br>$y$ -inter $p = 0.033$        | Slope $p = 0.127$<br>$y$ -inter $p = 0.332$        | Slope $p = 0.251$<br>$y$ -inter $p = 0.009$        |                    |

Source: Pavlovič, A., et al., *Bull. Environ. Contam. Toxicol.*, 77, 763, 2006. With permission.

Note: The regression lines for cv. Goral and for cv. Lutea had similar slopes ( $p > 0.01$ ), mean  $\pm$  SE,  $n = 20$ .

<sup>a</sup> Cd concentration in solution was log transformed.

**TABLE 24.8**  
**Values of Net Photosynthetic rate ( $P_N$ ) and Dark Respiration Rate ( $R_D$ ), Dry Weight, Root Length, and Cd Accumulation in the Plants Used for Photosynthetic Measurement (Mean  $\pm$  SE)**

| Parameter                                                                | Variant       | cv. Goral          | cv. Lutea                      |
|--------------------------------------------------------------------------|---------------|--------------------|--------------------------------|
| $P_N$ (nmol CO <sub>2</sub> g <sup>-1</sup> d.w. s <sup>-1</sup> )       | Control       | 148.48 $\pm$ 4.77  | 177.39 $\pm$ 8.63 <sup>▲</sup> |
| $n = 4$                                                                  | Cd 12 $\mu$ M | 110.12 $\pm$ 8.27* | 124.85 $\pm$ 10.91**           |
| $R_D$ shoot (nmol CO <sub>2</sub> g <sup>-1</sup> d.w. s <sup>-1</sup> ) | Control       | 34.22 $\pm$ 1.45   | 38.62 $\pm$ 1.59               |
| $n = 4$                                                                  | Cd 12 $\mu$ M | 36.75 $\pm$ 3.03   | 40.61 $\pm$ 3.81               |
| $R_D$ root (nmol CO <sub>2</sub> g <sup>-1</sup> d.w. s <sup>-1</sup> )  | Control       | 132.36 $\pm$ 21.43 | 44.19 $\pm$ 5.12 <sup>▲</sup>  |
| $n = 4$                                                                  | Cd 12 $\mu$ M | 181.87 $\pm$ 22.42 | 55.83 $\pm$ 9.60 <sup>▲▲</sup> |
| Shoot biomass (mg)                                                       | Control       | 116.92 $\pm$ 11.61 | 136.92 $\pm$ 8.36              |
| $n = 15$                                                                 | Cd 12 $\mu$ M | 85.00 $\pm$ 11.81  | 101.18 $\pm$ 15.88             |
| Root biomass (mg)                                                        | Control       | 25.86 $\pm$ 3.08   | 24.56 $\pm$ 2.77               |
| $n = 15$                                                                 | Cd 12 $\mu$ M | 15.44 $\pm$ 2.41*  | 12.33 $\pm$ 1.83**             |
| Root length (cm)                                                         | Control       | 14.14 $\pm$ 1.07   | 13.97 $\pm$ 0.81               |
| $n = 15$                                                                 | Cd 12 $\mu$ M | 10.50 $\pm$ 0.84*  | 9.65 $\pm$ 1.11**              |
| Shoot Cd content ( $\mu$ g g <sup>-1</sup> d.w.)                         | Control       | 15.9               | 4.61                           |
|                                                                          | Cd 12 $\mu$ M | 360.5              | 248.3                          |
| Root Cd content ( $\mu$ g g <sup>-1</sup> d.w.)                          | Control       | 19.9               | 44.8                           |
|                                                                          | Cd 12 $\mu$ M | 1081.0             | 895.0                          |

Source: Pavlovič, A. et al., *Bull. Environ. Contam. Toxicol.*, 77, 763, 2006. With permission.

Note: Comparisons were done between the control variant and Cd treatment (Cd 12  $\mu$ M) at  $p = 0.05$  (\*) and  $p = 0.01$  (\*\*), and between cultivars at  $p = 0.05$  (▲) and  $p = 0.01$  (▲▲). Student's  $t$ -test was used.

chlorophyll concentration), and production parameters (shoot and root biomass, shoot: root ratio, and lengths of shoots and roots) of young plants of *Hypericum perforatum* and *Chamomilla recutita* that were investigated. As the applied metal concentrations did not significantly affect the studied parameters (except for the root respiration rate), we can conclude that both investigated medicinal plants could be used in phytoextraction and the subsequent remediation of soils that are contaminated with cadmium and zinc.

Jakovljevic et al. [210] investigated the influence of the different doses of sodium selenate (0, 100, and 500 g Se per ha) applied by foliar spraying on the yield and quality of chamomile (*Chamomilla recutita* (L.) Rausch.—*M. recutita* L.). The applied doses of Se did not influence the formation of dry chamomile flowers' yield and the content of essential oil. However, the applied Se caused a significant increase in the content of bisabolol oxide A and B, followed by the decrease of the chamazulene content in the chamomile essential oil. Significant increase of Se content in the chamomile flowers (12.9–53.6 ppm) has also been observed. In our experiments with hydroponically cultivated chamomile plants, cv. Lutea [211] and cv Goral [212] treated with CdSeO<sub>4</sub>, CdSeO<sub>3</sub>, and Cd(NCSe)<sub>2</sub>(nia) we investigated Cd accumulation in the roots and shoots of plants (Table 24.9). The highest applied CdSeO<sub>3</sub> concentration (60  $\mu$ mol dm<sup>-3</sup>) caused a higher Cd content in the roots than the amount observed by employing CdSeO<sub>4</sub>, whereas in the case of treatment with 12 and 24  $\mu$ mol dm<sup>-3</sup> solutions of Cd(II) selenite and selenate adverse effects were observed. This could be correlated with the damage of the root cell membrane system due to the high concentration of cadmium and selenite ions. Immobilization of Cd ions in root tissue was manifested by a large amount of bioaccumulated Cd in this plant organ. The highest Cd content in the shoots was observed after the application

**TABLE 24.9**  
**The Amount of Accumulated Metal in Dry Mass of Roots and Shoots**  
**of *M. recutita* (cv. Goral) Plants**

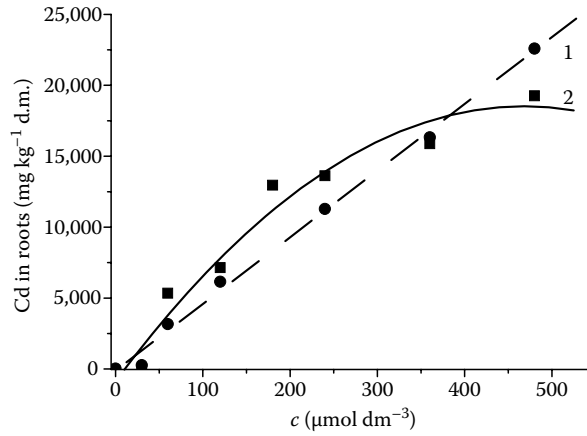
| Compound                                 | c<br>μmol dm <sup>-3</sup> | w (Cd)<br>(g kg <sup>-1</sup> ) |       | BAF  |       | TF   | Fraction of Cd<br>in Shoot (%) |
|------------------------------------------|----------------------------|---------------------------------|-------|------|-------|------|--------------------------------|
|                                          |                            | Root                            | Shoot | Root | Shoot |      |                                |
| Control                                  | 0                          | 0.02                            | 0.01  | —    | —     | 1.62 | 61.9                           |
| CdSeO <sub>3</sub>                       | 12                         | 0.75                            | 0.07  | 555  | 54.4  | 0.20 | 16.6                           |
|                                          | 24                         | 1.10                            | 0.07  | 408  | 27.2  | 0.25 | 19.8                           |
|                                          | 60                         | 4.93                            | 0.19  | 731  | 28.7  | 0.16 | 13.2                           |
|                                          | 12                         | 1.17                            | 0.21  | 863  | 155   | 0.60 | 37.6                           |
| CdSeO <sub>4</sub>                       | 24                         | 1.66                            | 0.36  | 613  | 133   | 0.68 | 40.5                           |
|                                          | 60                         | 1.85                            | 0.48  | 280  | 70.7  | 0.93 | 48.3                           |
|                                          | 12                         | 0.85                            | 0.07  | 633  | 48.6  | 0.27 | 21.0                           |
|                                          | 24                         | 1.23                            | 0.09  | 457  | 35.0  | 0.26 | 20.5                           |
| Cd(NCSe) <sub>2</sub> (nia) <sub>2</sub> | 60                         | 5.12                            | 0.17  | 759  | 25.1  | 0.12 | 10.5                           |
|                                          | 12                         | 0.79                            | 0.14  | 584  | 90.5  | 0.60 | 37.4                           |
|                                          | 24                         | 1.21                            | 0.23  | 449  | 84.0  | 0.70 | 41.2                           |
|                                          | 60                         | 2.10                            | 0.41  | 311  | 60.3  | 0.63 | 38.5                           |

Source: Král'ová, K. et al., *Chem. Pap.*, 61, 171, 2007. With permission.  
Note: Corresponding values of bioaccumulation and translocation factors and fraction of accumulated Cd allocated in shoots related to the total amount of Cd accumulated by the plant.

of CdSeO<sub>4</sub>. The change of S to Se in the complex Cd(NCX)<sub>2</sub>(nia)<sub>2</sub> led to a increase of Cd content in the shoots. In general, the content of Cd accumulated in plant organs after the application of Cd(NCSe)<sub>2</sub>(nia)<sub>2</sub> was comparable with that observed after the application of CdSeO<sub>3</sub>. The values of the translocation factor for Cd estimated for the experiments with CdSeO<sub>4</sub> and Cd(NCS)<sub>2</sub>(nia)<sub>2</sub> were more than two times higher than those found for the CdSeO<sub>3</sub> and Cd(NCSe)<sub>2</sub>(nia)<sub>2</sub>. The highest fraction of Cd accumulated in the shoots was observed for CdSeO<sub>4</sub>, while the lowest fraction observed was for Cd(NCSe)<sub>2</sub>(nia)<sub>2</sub>. The obtained results correspond with those obtained for the chamomile cultivar Goral [212] that was found to be more tolerant to the cadmium exposure compared to the cultivar Lutea [211]. The treatment with CdSeO<sub>4</sub> and Cd(NCS)<sub>2</sub>(nia)<sub>2</sub> caused approximately 40% of the total amount of Cd that was accumulated by the plant in its shoots. On the other hand, approximately 80% (or more) from the total amount of the metal accumulated by the plant remain in the roots after the treatment with CdSeO<sub>3</sub> and Cd(NCSe)<sub>2</sub>(nia)<sub>2</sub>. These data correlate well with the results of Shanker et al. [213]. The observed fact could be explained by taking into account the fact that the less mobile selenite after being reduced to the selenide tends to form Cd–Se complex, which appears to be unavailable for the plants. On the other hand, the more mobile anion selenate is available for Cd–Se formation only after following a more complicated redox process involving Se(VI) in SeO<sub>4</sub><sup>2-</sup>, Se(IV) in SeO<sub>3</sub><sup>2-</sup>, and Se(0) species. According to Whanger [214], the presumed protective effect of Se against cadmium and mercury toxicity is through the diversion in their binding from low-molecular-mass proteins to higher-molecular-mass ones. The experiments with chamomile cv. Goral also showed that the BAF values related to Se accumulation in plant organs were significantly influenced by the oxidation state of Se—the application of selenate resulted in an intensive translocation of Se into the shoots and for this compound the BAF values determined for shoots were approximately 2.5 times higher than those determined for roots. The corresponding BAF values for Cd(NCSe)<sub>2</sub>(nia)<sub>2</sub> were similar to those of CdSeO<sub>3</sub>. At application of CdSeO<sub>4</sub>, 90% of the uptaken Se and 50% of the uptaken Cd was situated in the shoots [212].

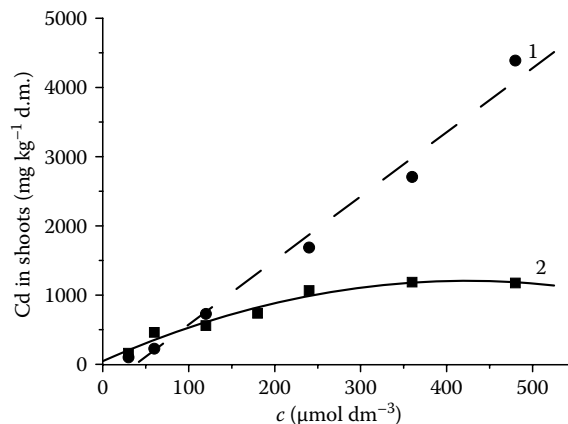
The presence of chelators can alter the mobility and transport of Zn, Cd, and Ni in soils because of the formation of water-soluble chelates, thus increasing the potential for the metal pollution of natural waters. However, chelators could also increase the bioavailability and uptake of toxic metals. Chelated metals are taken up via the apoplastic pathway. Disruption of the Casparian band is required to achieve the high-shoot concentrations that are needed for phytoextraction. Therefore, adding chelators to a soil increases not only the total dissolved metal concentration but also changes the primary route of the plant metal-uptake from the symplastic to the apoplastic pathway and depending on metal, plant species, and chelant concentration, significant increases in the metal uptake are likely [215]. The addition of an organic chelator (citric acid) enhanced the zinc and cadmium accumulation, mostly at the root level [216]. Our experiments with a set of Cu(II) chelates confirmed that the application of copper in the form of chelates led to more effective Cu translocation into the shoots in comparison to  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  treatment [217]. Artificial chelator ethylenediaminetetraacetic acid (EDTA) promoted Cu translocation into the shoots of hydroponically cultivated chamomile plants very effectively which was reflected in the fact that at the treatment with higher metal concentrations (24 and  $60 \mu\text{mol dm}^{-3}$ ) even 45% and 59% Cu accumulated by plants was allocated in the shoots (in the absence of a chelator this portion reached only 5.2% and 4.8%, respectively). Very efficient translocation of copper into the shoots observed at the presence of EDTA could be connected with the largest value of Cu-EDTA stability constant ( $\log K_1 = 18.8$ ). Whereas the chelate formation between the EDTA and Zn or Cu resulted in a significantly decreased metal uptake into the chamomile roots, the decrease of Cd uptake due to chelate formation was very low [218].

*Salvia officinalis* L. is in general also one of the most important medicinal and aromatic plants with a wide spectrum of application in phytotherapy, cosmetics, and food industry. The genus *Salvia* includes more than 400 species. *S. officinalis* as a perennial plant originates from the Mediterranean region. Concerning the analysis of sage essential oil, the major compounds are thujone, cineole, camphor, and caryophyllene. These secondary metabolites are biologically active compounds present in *Herba salviae* with applications in phytotherapy. In the food industry, this aromatic plant species is recommended as a spice or as an additive substance (cf. [219,220]). From all the above-mentioned aspects, it is important to have information of toxic metal effects on growth and metal accumulation into the different plant organs of this species. Since Marquard and Schneider [193] characterized *S. officinalis* as the excluder of cadmium, we studied the effect of large external concentration ranges of cadmium ( $30\text{--}480 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ ) on the production characteristics (length of roots and shoots as well as dry mass of roots and shoots) of this species. We tested two cultivars: cv. Krajova (Slovakian provenance) and cv. Primorska (Yugoslavian provenance). Two months old plants were cultivated hydroponically for 7 days under controlled conditions in a Hoagland solution, without and in the presence of  $\text{Cd}(\text{NO}_3)_2$  [221]. The plants were exposed in hydroponia for 7 days in controlled conditions: control variant in Hoagland solution and metal treated variants in Hoagland solution with 30, 60, 120, 240, 360, and  $480 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ . Then the length and dry mass of the shoots and roots were estimated. Metal content in aboveground and underground parts of the studied species were determined using FAAS. Differences were found in the phenology and production parameters between the two tested cultivars of different provenance. cv. Krajova was already sensitive to the concentration of  $60 \mu\text{mol dm}^{-3}$  of  $\text{Cd}(\text{NO}_3)_2$  when the oldest leaves dried. At a concentration of  $120 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ , all the older leaves were dried and the younger leaves wilted. At  $240 \mu\text{mol dm}^{-3}$  of  $\text{Cd}(\text{NO}_3)_2$ , brown spots were observed on the leaves, and at the applied highest metal concentrations of 360 and  $480 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ , all the leaves were dried and on the apical side of the leaves depigmentation was observed. Cultivar Primorska seems to be more tolerant to metal treatment. Visual changes occurred up to  $120 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$  when only some of older leaves of the plant dried up. At the concentration of  $240 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ , the leaves were dried but they were green colored. This fact confirms the disturbance of the water regime and indicates strong water stress. At the highest tested Cd concentrations (360 and  $480 \mu\text{mol dm}^{-3}$   $\text{Cd}(\text{NO}_3)_2$ ) all the leaves dried and the damage of leaf pigmentation was observed as the brown colored spots. In spite of the high concentration of Cd ( $30\text{--}480 \mu\text{mol dm}^{-3}$ ), the length of the roots



**FIGURE 24.11** Dependence of Cd content in roots of *Salvia officinalis* plants on the concentration of  $\text{Cd}(\text{NO}_3)_2$  in the hydroponics (1, cv. Primorska; 2, cv. Krajova).

in both cultivars was almost not influenced. Also for the shoots of both cultivars, only a slight reduction of length was found. On the other hand, the dry mass of the shoots decreased at all applied Cd concentrations more expressively than the dry mass of the roots. The negative effect of the high Cd concentrations on the shoot dry mass was manifested mainly in cv. Krajova. The greatest portion of Cd into the roots was uptaken by both the cultivars (Figure 24.11). cv. Primorska accumulated two times more Cd than cv. Krajova in the shoot app. (Figure 24.12). However, the differences were found in the translocation of the cadmium from the roots into the shoots. cv. Krajova did not allocate the Cd from the roots into the shoots already at  $240 \mu\text{mol dm}^{-3} \text{Cd}(\text{NO}_3)_2$ , which confirms the existence of some barriers in the roots. Bioaccumulation factor (BAF) for root depending on Cd concentration ranged from 565 to 357 and for shoot it was 47 to 22. Percent of Cd in the shoot was 27.8–29.0. On the other hand, for cv. Primorska, at increasing applied Cd concentrations, the Cd translocation from the roots into the shoots increased in the whole applied Cd concentration range. BAF for the root ranged from 817 to 419 and for the shoot from 30 to 81. The percent of Cd in the shoot was 9.4–41.0. On the basis of the results and definition of the Cd hyperaccumulators [222], it could be concluded that both studied cultivars belong to the category of hyperaccumulators of cadmium because they accumulated more than  $100 \mu\text{g g}^{-1}$  of Cd in the shoots. However, cv. Primorska is more effective mainly for the translocation of cadmium into the aboveground part of plants. Our



**FIGURE 24.12** Dependence of Cd content in shoots of *Salvia officinalis* plants on the concentration of  $\text{Cd}(\text{NO}_3)_2$  in the hydroponics (1, cv. Primorska; 2, cv. Krajova).



findings for both the studied cultivars of *Salvia officinalis*, cv. Primorska and cv. Krajova, did not support the results of Marquard and Schneider [193], which characterized this medicinal plant species as an excluder of Cd.

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# 25 Heavy Metal Pollution: Damage and Defense Strategies in Plants

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## 25.1 INTRODUCTION

In the literature the term “heavy metal” is used with a very broad and misleading meaning but in a strict sense it includes a group of metals with density higher than  $5.0 \text{ g cm}^{-3}$  and an atomic weight above 20. Actually, there is a strong tendency to classify metals according to their propensity to interact with biological ligands, which largely defines their toxicity. This chapter speaks of heavy metals as metals and metalloids that are toxic to plants.

Phytotoxic amounts of heavy metals are occasionally found in soils under natural conditions but more frequently they are mobilized and released by technological and agricultural activities and tend to persist indefinitely, circulating and eventually accumulating throughout the food chain, thus posing a serious threat to animals, humans, and the environment. In Europe, the polluted agricultural lands likely encompass several million hectares (Flathman and Lanza, 1998). Some heavy metals (Mn, Fe; Cu, Zn, Mo, and Ni) are essential nutrients necessary for the normal growth of plants but

when present at supra-optimal concentrations they can easily become phytotoxic. Of major concern with respect to plant exposure as well as accumulation in the human food chain are the metalloid arsenic (As), and the metals cadmium (Cd), mercury (Hg), and lead (Pb) (McLaughlin et al., 1999). The similarity to essential elements makes these nonessential elements potentially toxic to plants. In the environment, plants cannot move and can encounter elevated levels of both essential and nonessential metals much more than other organisms. Toxicity symptoms may derive from interactions at cellular/molecular level, such as blocking functional groups of biologically important molecules, displacing and/or substituting essential elements, inactivating enzymes, and disrupting cell and organelle membrane integrity.

The high amount of metals in the tissue of hyperaccumulator plants suggests the existence of defense mechanisms to avoid the harmful effects caused by metals. These mechanisms are quite complex and their importance may vary in accordance with the metal, its concentration, the species, and even the plant organs and stages of development in the same plant, etc.

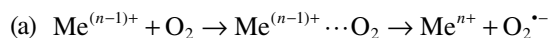
This chapter focuses on responses of plants to toxic metals and the metalloid As exposure, and on mechanisms of tolerance that help plants to maintain growth avoiding interferences with the normal cellular metabolism.

## 25.2 HEAVY METAL TOXICITY IN PLANTS

At low metal concentrations the plant cell can resort to a number of avoidance mechanisms such as metal exclusion, translocation, and complexation in the cytoplasm (Vansgrovel and Clijsters, 1994). At high concentrations, when primary barriers are broken down, avoidance is insufficient, free metal concentration increases, and both redox and nonredox metals can stimulate production of reactive oxygen species (ROS) imposing oxidative stress (Aust et al., 1998; Navari-Izzo et al., 1998, 1999; Quartacci et al., 2001). Although  $O_2$  itself is not a harmful molecule, it can potentially be reduced to form toxic ROS. In the plant system, including algae, ROS are always formed by the inevitable leakage of electrons into molecular oxygen from the electron transport activities of chloroplasts, mitochondria, and plasma membranes. A growing body of evidence indicates that various toxic metals act as catalyst in the oxidative deterioration of biological macromolecules, and therefore the toxicities associated with these metals may be due, at least in part, to oxidative damage to the tissues. Actually, metals such as Cr, Cu, Fe, Hg, Ni, Pb, and V exhibit the ability to increase the normal production of ROS, resulting in lipid peroxidation (Quartacci et al., 2001), DNA damage, depletion of sulfhydryl groups, and altered calcium homeostasis (Stohs and Bagchi, 1995). There is significant evidence that exposure to the metalloid As also enhances the production of ROS, leading to membrane damage through the peroxidation of membrane lipids (Hartley-Whitaker et al., 2001a).

### 25.2.1 REACTIVE OXYGEN SPECIES PRODUCTION

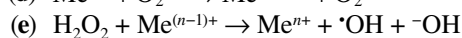
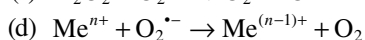
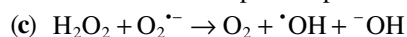
Redox metals, performing one-electron oxidation reactions, can easily catalyze the formation of free radical through a Fenton-type reaction (e):



In aqueous solutions at neutral pH,  $O_2^{\bullet -}$  can generate  $H_2O_2$



which can decompose to produce the Haber-Weiss reaction (c):



Cycling of redox active metals, such as Cu and Fe, at/or near the binding sites of cell membrane constituents may also lead to a site-specific production of hydroxyl radicals ( $\bullet OH$ ) via Haber-Weiss

reaction (c) (Chevion, 1988). In PSII isolated from wheat grown in excess Cu, the production of harmful oxygen species, such as hydroxyl radicals arising from superoxide has been demonstrated (Navari-Izzo et al., 1998, 1999). However, the kind of free radicals involved in DNA-strand cleavage is still a controversial problem. In fact, some authors suggested that singlet oxygen rather than hydroxyl radical may play a role in the induction of DNA strand breaks (Li and Trush, 1993) while others reported the involvement of both forms (Yamamoto and Kawanishi, 1989). As  $\text{Hg}^{2+}$  cannot replace  $\text{Cu}^+$  or  $\text{Fe}^{2+}$  in the Fenton reaction a different mechanism should cause an accumulation of ROS (Cho and Park, 2000; Patra et al., 2004; Han et al., 2007). The  $\text{Hg}^{2+}$  ions bind avidly to  $-\text{SH}$  groups and likely inhibit the activities of antioxidative enzymes especially of glutathione reductase (GR), and also raise a transient depletion of reduced glutathione (GSH) (Lomonte et al., 2010). Since glutathione is of pivotal importance for the redox status of the cells (Foyer et al., 1997) it may be guessed that a short-term depletion of GSH would have as consequence a natural accumulation of ROS. Mercury also can displace  $\text{Cu}^{2+}$  ions from metallothioneins *in vitro*, which might potentiate oxidative damage if it occurs *in vivo*. The question rises whether nonredox metals, which are incapable of univalent oxireduction reactions, are capable of producing ROS. In animal tissues, it has been demonstrated that Cd induces changes in the antioxidant status by either increasing superoxide radical production and lipid peroxidation or by decreasing enzymatic and nonenzymatic antioxidant levels (Stohs and Bagchi, 1995). Evidences have been reported that Cd leads to oxidative stress acquired in tolerant and sensitive clones of *Holcus lanatus* (Hendry et al., 1992) and in germinating seedlings of *Phaseolus vulgaris* (Somashekaraiiah et al., 1992). In contrast with other heavy metals such as Cu or Fe, Cd does not directly produce ROS via Fenton and/or Haber–Weiss reactions but the production of ROS in plants may result from the activity of redox enzymes bound or associated to the plasma membrane of the cells. The induction of lipoxygenase (LOX, EC 1.13.11.12) in the presence of nonredox metals might explain its effect on the cellular redox status possibly related to the ability of this enzyme to produce superoxide anion ( $\text{O}_2^{\bullet-}$ ) by oxidizing NADPH (Roy et al., 1994; Quartacci et al., 2001). LOX mediates polyunsaturated fatty acid oxidation and produces free radicals from fatty acids which in turn cause the destruction of the membranes. The iron-containing LOX is known to initiate lipid peroxidation (Thompson et al., 1987), and a high level of Zn promotes free radical generation and hence peroxidative degradation of polyunsaturated fatty acids (Weckx and Clijsters, 1997). The increase in free radical production could be due to interference of Zn with the normal functioning of electron transport chains in mitochondria and chloroplasts. In fact, heavy metals, including Zn, have been reported to suppress electron transport chain associated with these organelles (Weckx and Clijsters, 1997). Excess of metal ions blocks the electron flow in PSII (Pagliano et al., 2006) which leads to the formation of excess energy which in its turn causes production of ROS. Therefore, damage to biomembranes by lipid peroxidation might not be limited to redox metal only.

Recently, it became evident that ROS play a dual role in plants both as toxic compounds and as key regulators of many biological processes. The identification of ROS-generating enzymes such as plant homolog of respiratory burst NADPH oxidase (Rboh) demonstrates that plant cells can initiate and most likely amplify ROS production for signaling (Miller et al., 2008). An oxidative burst can be observed in plants as an early response to pathogen attack (Frahry and Schopfer, 2001).  $\text{O}_2^{\bullet-}$ , released in the apoplast by NAD(P)H oxidase, dismutates to  $\text{H}_2\text{O}_2$  spontaneously and this reaction is enhanced by superoxide dismutase (SOD, EC 1.15.1.1) that has been also shown to be present in this compartment (Ogawa et al., 1996). The release of  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  in the apoplastic space is believed to contribute to several disease resistance strategies in plants. ROS, and, in particular  $\text{H}_2\text{O}_2$ , have been considered as signal molecules in the environmental stress response since they induce the expression of a variety of defense-related genes (Foyer et al., 1997). However, very little is known in the case of excess of metals. In wheat seedlings, excess Cu induced a biphasic increase of  $\text{O}_2^{\bullet-}$  in the apoplast. A very high SOD activity in the apoplast dismutated the superoxide anion giving rise to an increase in  $\text{H}_2\text{O}_2$ . The highest value of  $\text{H}_2\text{O}_2$  was detected at 15 min of Cu exposure when peroxidase (POD, EC 1.11.1.9) activity reached the lowest value (Sgherri et al., 2007). Furthermore, in

*Brassica napus*, a link between lipid signals and redox antioxidative signals was found as an early response to a treatment with excess Cu (Russo et al., 2008).

## 25.3 METAL TOLERANCE

In order to minimize the negative effects of nonessential toxic metal and to maintain the concentration of essential metals within physiological limits, plants, as have other organisms, have evolved a range of homeostatic mechanisms to control the uptake, accumulation, and transport of the metals to possess a basic metal tolerance (Clemens, 2001). Only certain plants species and genotypes possess a naturally selected hyper tolerance toward particular metals different from the basal tolerance common to all plant species and varieties. Metal hypertolerance or tolerance in plants is the ability of certain plants to survive and reproduce in metal-rich soils with concentrations of metals toxic to other plants (MacNair and Baker, 1994), which is different from the basic normal tolerance held by all plants (Clemens, 2006). Basic strategies include exclusion, efflux of toxic metal ions, immobilization, compartmentalization and metal chelation, reduction of metal transport, and expression of other general stress response mechanisms. In addition, metallothioneins (MT<sub>s</sub>) and phytochelatins (PC<sub>s</sub>) are molecular components which play a significant role in tolerance.

In metal tolerance test root growth is the most widely used parameter and the quantification of tolerance index (TI) in plants has been based on the use of relative measurements of the rates of root growth of plants tested in control and in metal-exposed plants (Wilkins, 1978; Baker, 1987). On this basis, a tolerance index (TI) can be calculated:

$$TI (\%) = \frac{\text{root growth in solution with metal}}{\text{root growth in solution without metal}}$$

However, there are some limitations for the use of TI (%) (MacNair, 1981, 1983; Verkleij and Prast, 1989; Schat and Ten Bookum, 1992).

An important generalization is that due to the distinct chemical properties of the various metals, the tolerance machineries are essentially metal-specific.

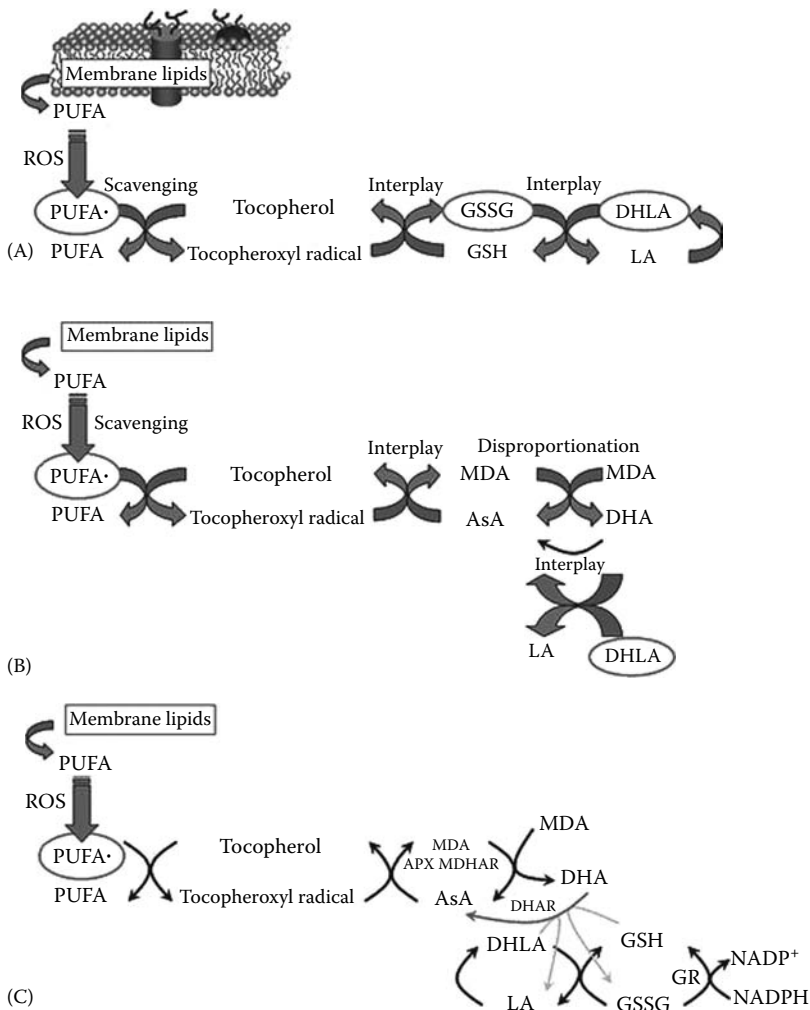
Higher plants employ two basic strategies to tolerate heavy metals: (1) avoidance or exclusion, which restricts the uptake and/or root to shoot transport; and (2) accumulation and sequestration, which allow plants to survive accumulation and detoxify metals in the shoots by compartmentation of metals in the vacuole, by complexation of metals by organic ligands such as organic acids, amino acids, and metal-binding peptides (Baker, 1981; Clemens, 2001; Hall, 2002). Metal exclusion is by far the most common strategy in metal tolerant species. De Vos et al. (1991) compared copper uptake in roots of two copper-tolerant *Silene vulgaris* populations from copper mines and a copper sensitive population from a nonmetalliferous site. The net copper uptake at the same external copper concentration was found to be inversely related to the copper tolerance level. This suggests that a reduced uptake and/or an increased efflux of copper across the root plasma membrane might contribute to the higher tolerance of the mine plants. However, if compared at their own no-effect concentration (NEC), 50%-effect concentration (EC50) or 100%-effect concentration (EC100) values, this relationship is reversed meaning that the maximum tolerable Cu uptake rates are manifold higher in hypertolerant *S. vulgaris* than in sensitive one (Schat and Kalff, 1992). Therefore, adaptive Cu hypertolerance must rely on an increased capacity to sequester the metals inside the plant (Verkleij, 2008). On the other hand, metal accumulation is a rare phenomenon on terrestrial higher plants. To date, about 450 plants have been identified as metal hyperaccumulators, representing less than 0.2% of all angiosperms.

### 25.3.1 MECHANISMS OF METAL TOLERANCE IN PLANTS

#### 25.3.1.1 Cellular Antioxidant Defense against Oxidative Stress Induced by Metals

The induction of the activity of particular groups of enzymes as well as reducing metabolites is considered to play an important role in the cellular defense strategy against oxidative stress.

Hydrophilic antioxidants such as reduced glutathione (GSH) and ascorbate (AsA) participate in the defense in the aqueous phase, while the lipophilic tocopherols and carotenoids fulfill essential antioxidant action in membranes. These antioxidants can be regenerated through the glutathione-ascorbate cycle which utilizes NADPH as reducing agent (Sgherri and Navari-Izzo, 1995). In addition, lipoic acid (LA), due to its solubility in both water and lipid phases, connects the activity of antioxidants in the cell membrane (tocopherols) with antioxidants in the cytoplasm (AsA and GSH), strengthening the antioxidant network (Navari-Izzo et al., 2002; Sgherri et al., 2002) (Figure 25.1). LA is unique among antioxidant molecules in that it retains protective functions in both its reduced (DHLA) and oxidized forms (LA) although DHLA is the more effective in performing antioxidant functions (Navari-Izzo et al., 2002). DHLA acts directly by scavenging  $O_2^{\cdot-}$ , hydroperoxyl, and hydroxyl radicals and can also donate an electron to oxidised glutathione (GSSG) and dehydroascorbate (DHA).



**FIGURE 25.1** Diagrams showing the scavenging of lipid peroxyl radicals formed in membrane, the regeneration of tocopheroxyl radical by glutathione (A) or ascorbate (B) and the interplay of lipoic acid in the regeneration of ascorbate and glutathione (C). APX, ascorbate peroxidase; AsA, reduced ascorbate; DHAR, dehydroascorbate reductase; DHA, dehydroascorbate; DHLA, dehydrolipoic acid; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; LA, lipoic acid; MDA, monodehydroascorbate; MDHRA, monodehydroascorbate reductase; PUFA, polyunsaturated fatty acids; PUFA $\cdot$ , lipid peroxyl radical.

SOD, (EC 1.15.1.1), the first enzyme in the detoxification process, converts  $O_2^{\bullet-}$  to  $H_2O_2$ . Catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.7) and a variety of general peroxidases catalyze the breakdown of  $H_2O_2$  (Navari-Izzo et al., 1997; Navari-Izzo and Rascio, 1999). To regenerate oxidized ascorbate, plant cells use monodehydroascorbate reductase (MDHA, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2). Glutathione peroxidases (GP<sub>s</sub>, EC 1.11.1.9) and glutathione transferases (GST<sub>s</sub>, EC 2.5.1.18), also contribute to the redox status maintenance of the cells. Under normal conditions the balance between the generation of ROS and the mechanisms that protect cells from the action of ROS is tightly controlled, but in presence of metal excess an increase in the level of ROS and a decrease in the detoxification mechanisms can lead to degradation of biomembranes (Quartacci et al., 2000, 2001; Berglund et al., 2002; Calucci et al., 2003). In the presence of excess metals, cells may attempt to prevent the formation of ROS by increasing the production of metal-binding compounds and/or by scavenging them via antioxidative defense systems. In hyperaccumulator plants evidences for a role of antioxidative defenses are scanty and do not provide a basis sufficient for defining direct relationships (Cuypers et al., 1999; Schickler and Caspi, 1999). In recent years however, in metal-accumulator species, the activities of SOD, CAT, and POD were shown to be enhanced upon Cd exposure (Mobin and Khan, 2007; Sun et al., 2007a,b) and Wang and collaborators (2008) found that compared to tobacco plants, metal accumulator plants are equipped with superior antioxidative defense to adapt to the oxidative stress induced by Cd toxicity. Overexpression of enzymes involved in antioxidative defenses seems a useful approach to obtain metal tolerance. The transformed accumulator Indian mustard (*Brassica juncea*) overexpressing glutathione synthetase increased the biosynthesis of glutathione enhancing Cd tolerance and accumulation more than the nontransformed plants (Zhu et al., 1999), while GR overexpression in the plastids led to increased Cd tolerance at chloroplast level but decreased GSH accumulation in the shoots (Pilon-Smith et al., 2000). In *Arabidopsis* the overexpression of glutathione transferase and peroxidase resulted in enhanced Al tolerance (Ezaki et al., 2000), and increased transcript levels of APX, CAT, and GP in *Hordeum vulgare* subjected to Cd stress have been reported (Finkemeier et al., 2003; Metwally et al., 2003; Sharma et al., 2004). There are conflicting reports about nonhyperaccumulator plants; these reports depend on the plant species and tissue analyzed, growth conditions, the metal used for the treatment and its concentrations as well as the organelle taken into consideration (Table 25.1). Therefore, the observations reported throughout the text may depend on the experimental design and are likely to be element- and species-dependent. Subsymptomatic concentrations of Cu or Cd increased CAT activity in a sensitive cv of wheat, while a more tolerant cv responded with the induction of POD (Sgherri et al., 2001; Milone et al., 2003). Increase in CAT activity was also observed in pea plants grown for 18 and 33 days under toxic concentrations of Mn or Zn (Del Rio et al., 1985). In *P. vulgaris*, Cd induced POD but decreased CAT activities (Shaw, 1995). CAT activity decreased also in *Bacopa monnieri* where the activities of SOD and POD were instead enhanced by Cd (Singh et al., 2006). In pea the Mn-SOD and CAT of peroxisomes were found to be higher in Cu-tolerant than in Cu-sensitive plants, suggesting a protective role of these enzymes in Cu-induced oxidative stress in pea leaf peroxisomes (Palma et al., 1987). On the contrary, in pea plants both SOD and CAT activities as well as accumulation of transcripts were decreased by Cd, while POD did not change (Romero-Puertas et al., 2007). However, in *Solanum nigrum* the activities of the three enzymes were enhanced by Cd exposure (Mobin and Khan, 2007; Sun et al., 2007) suggesting species-specific differences as response to Cd toxicity. In both thylakoids and PSII particles of wheat treated with 50  $\mu$ M Cu, a higher production of superoxide was monitored together with an induction in thylakoid-bound CuZn-SOD and APX. The activity of the stromal enzymes SOD, APX, and MDHA was also increased, thus suggesting that Cu might have induced a general response in the biosynthesis of both thylakoids and stromal enzymes by directly influencing their gene expression (Navari-Izzo et al., 1998). In this way,  $O_2^{\bullet-}$  and  $H_2O_2$  could be efficiently removed, and possible formation of  $\cdot$ OH, highly toxic for biological membranes, avoided. In *Helianthus annuus* Fe, Cu or Cd decreased the activity of CAT, APX, GR,



**TABLE 25.1**  
**Trend of Activities of Antioxidative Enzymes in Different Plant Species Subjected to Toxic Metals**

| Metal | Species                                      | Enzyme Activity <sup>a</sup>                                              | References                      |
|-------|----------------------------------------------|---------------------------------------------------------------------------|---------------------------------|
| As    | <i>Holcus lanatus</i> (As-tolerant)          | SOD (+)                                                                   | Hartley-Whitaker (2001)         |
|       | <i>Holcus lanatus</i> (As-sensitive)         | SOD (–)                                                                   | Hartley-Whitaker (2001)         |
|       | <i>Hydrilla verticillata</i>                 | APX (+), CAT (+), GPX (+), GR (+), SOD (+)                                | Srivastava et al. (2007)        |
|       | <i>Nephrolepis exaltata</i>                  | APX (=), CAT (+), GPX (+), GR (+), SOD (+)                                | Srivastava et al. (2005)        |
|       | <i>Pteris eusiformis</i>                     | APX (+), CAT (+), GPX (+), GR (+), SOD (+)                                | Srivastava et al. (2005)        |
|       | <i>Pteris vittata</i>                        | APX (+), CAT (+), GPX (=), GR (=), SOD (+)                                | Srivastava et al. (2005)        |
| Cu    | <i>Brassica juncea</i>                       | APX (+), CAT (–), GPX (+), SOD (+)                                        | Wang et al. (2004)              |
|       | <i>Helianthus annuus</i>                     | APX (–), CAT (–), DHA (–), GR (–), SOD (+)                                | Gallego et al. (1996)           |
|       | <i>Triticum durum</i><br>(drought-sensitive) | APX (–), CAT (+), GPX (=), SOD (–)                                        | Sgherri et al. (2001)           |
|       | <i>Triticum durum</i><br>(drought-tolerant)  | APX (+), CAT (–), GPX (+), POD (+),<br>SOD (=)                            | Sgherri et al. (2001)           |
|       | <i>Triticum durum</i>                        | Stromal-APX (+), -MDHA (+), -SOD (+),<br>Thylakoid-APX (+), -Cu-ZnSOD (+) | Navari-Izzo et al. (1998)       |
|       |                                              |                                                                           |                                 |
| Cd    | <i>Alyssum argenteum</i>                     | APX (+), GR (–), SOD (+)                                                  | Schickler and Caspi (1999)      |
|       | <i>Bacopa mounieri</i>                       | CAT (–), POD (+), SOD (+)                                                 | Singh et al. (2006)             |
|       | <i>Brassica juncea</i>                       | CAT (+), SOD (+)                                                          | Wang et al. (2008)              |
|       | <i>Brassica juncea</i>                       | CAT (+), POD (+), SOD (+)                                                 | Mobin and Khan (2007)           |
|       | <i>Helianthus annuus</i>                     | APX (–), CAT (–), DHA (–), GR (–), POD (+),<br>SOD (–)                    | Gallego et al. (1996)           |
|       | <i>Nicotiana tabacum</i>                     | CAT (–), POD (+), SOD (+)                                                 | Wang et al. (2008)              |
|       | <i>Phaseolus vulgaris</i>                    | APX (+), CAT (–), GPX (+), GR (+), POD (+)                                | Chaoui et al. (1997)            |
|       | <i>Phaseolus vulgaris</i>                    | APX (+), CAT (–), GPX (+)                                                 | Shaw (1995)                     |
|       | <i>Pisum sativum</i>                         | CAT (–), POD (=), SOD (–)                                                 | Romero-Puertas et al.<br>(2007) |
|       | <i>Solanum nigrum</i>                        | CAT (+), POD (+), SOD (+)                                                 | Sun et al. (2007)               |
|       | <i>Thlaspi caerulescens</i>                  | CAT (+), SOD (+)                                                          | Wang et al. (2008)              |
|       | <i>Triticum durum</i><br>(drought-sensitive) | APX (–), CAT (+), GPX (=), SOD (–)                                        | Milone et al. (2003)            |
|       | <i>Triticum durum</i><br>(drought-tolerant)  | APX (+), CAT (+), GPX (+), SOD (=)                                        | Milone et al. (2003)            |
|       |                                              |                                                                           |                                 |
|       |                                              |                                                                           |                                 |
| Fe    | <i>Helianthus annuus</i>                     | APX (–), CAT (–), DHA (–), SOD (–)                                        | Gallego et al. (1996)           |
| Hg    | <i>Atriplex codonocarpa</i>                  | APX (+), GR (–), SOD (+)                                                  | Lomonte et al. (2010)           |
|       | <i>Lycopersicon esculentum</i>               | CAT (+), SOD (+), POD (=)                                                 | Cho and Park (2000)             |
|       | <i>Phaseolus aureus</i>                      | CAT (+), POD (+)                                                          | Shaw (1995)                     |
| Mn    | <i>Pisum sativum</i>                         | CAT (+)                                                                   | Del Rio et al. (1985)           |
| Ni    | <i>Alyssum argenteum</i>                     | APX (+), GR (+), SOD (–)                                                  | Schickler and Caspi (1999)      |
|       | <i>Alyssum maritimum</i>                     | APX (+), GR (+), SOD (+)                                                  | Schickler and Caspi (1999)      |
| Zn    | <i>Phaseolus vulgaris</i>                    | APX (+), CAT (–), GPX (+), GR (+), POD (+)                                | Chaoui et al. (1997)            |
|       | <i>Pisum sativum</i>                         | CAT (+)                                                                   | Del Rio et al. (1985)           |

Note: APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate reductase; GPX, guaiacol peroxidase; GR, glutathione reductase; MDHA, monodehydroascorbate reductase; POD peroxidase; SOD, superoxide dismutase.

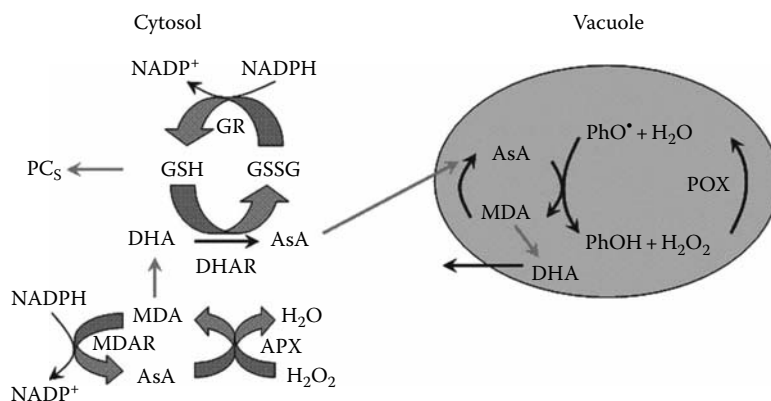
<sup>a</sup> In comparison to control: +, increase; –, decrease; =, no change.

and DHAR, while enhanced LOX activity, thus provoking lipid peroxidation. In the same plants, Fe and Cd ions caused a decrease in SOD activity, while Cu raised SOD levels (Gallego et al., 1996). Upon Cd exposure, lipid peroxidation was raised in pea plants (Lozano-Rodriguez et al., 1997), in *Phaseolus aureus* (Shaw, 1995) and in *P. vulgaris* (Chaoui et al., 1997), whereas in *Daucus carota* no lipid peroxidation was observed (Sanità di Toppi and Gabbrielli, 1999).

The enzymes of the ascorbate-glutathione pathway take part in the defense against oxidative stress as they increased after application to bean seedlings of Cu and Zn, metals with different chemical behavior, indicating that both induced oxidative stress (Cuypers et al., 1999). However, striking differences in the relative induction time of these enzymes suggest that the chemical properties of the metals play a pivotal role in the induction of oxidative stress as well as in the induction of defense mechanisms.

Some studies have been conducted to analyze induction of the antioxidant system under As stress (Hartley-Whitaker et al., 2001a; Srivastana et al., 2005). In *Pteris vittata* exposed to As, higher activities of antioxidant enzymes and lower peroxidation corresponded to a higher As hyperaccumulation and lack of toxicity symptoms in comparison to other ferns such as *P. ensiformis* and *Nephrrolepis exaltata* (Srivastava et al., 2005). Only a few studies compared the responses of antioxidants to As<sup>+3</sup> and As<sup>+5</sup> (Mylona et al., 1998; Srivastana et al., 2007). It appears that upon exposure of the plants to As<sup>+5</sup> SOD, APX, and GR activities were stimulated to higher levels compared to As<sup>+3</sup>, thus maintaining the function of the ascorbate-glutathione cycle. The increase in the activities of antioxidant enzymes may be due to the induced transcription of their genes mediated by ROS.

Varying responses are also likely related to concentration of –sulfhydryl (SH) groups present in the plants or induced by metal treatment. Thiols have strong antioxidative properties and it is important to know to what extent GSH is involved in metal tolerance. GSH not only directly reacts with free radicals and protects the thiol status of protein via thiol-disulfide exchange, but is also involved as substrate for GP, which reduces H<sub>2</sub>O<sub>2</sub> and organic peroxides, thus protecting cell proteins and cell membranes against oxidation (Navari-Izzo and Izzo, 1994). Biosynthesis of GSH was enhanced in the presence of Cd and other heavy metals, but a low GSH concentration was instead found in the plants (Rueggsegger and Brunold, 1994; Xiang and Oliver, 1998). These results are consistent with the idea that GSH, besides being itself an antioxidant and a metal chelator, is also a precursor for the synthesis of metal-binding phytochelatins. In addition, the oxidation of GSH in response to oxidative damage is also important for protection of plasma membrane from lipid peroxidation. The hypothesis that in *Nicotiana rustica* constitutive GSH might chelate Cd ions present in the cytosol under low-level exposure (5 µM) is intriguing (Vögeli-Lange and Wagner, 1990), but the drop in GSH observed in the first hours of exposure to Cd could be due to its utilization against oxidative stress caused by the metal. May be that higher levels of exposure require additional response mechanisms, like synthesis of PC<sub>s</sub>. A complex defense system, comprising of antioxidant enzymes and PC<sub>s</sub>, occurred in the roots of *Lupinus luteus* exposed to Ca, Cu, and Pb (Gwozdz et al., 1997). In *Raphanus sativus* subjected to increased concentration of Cu or Cd, total glutathione (GSH + GSSG) did not increase, whereas oxidized glutathione (GSSG) rose with the increase in metal concentration (Cosi, 2001; Sgherri et al., 2003), indicating an enhancement of oxidative processes and an involvement of GSH in counteracting oxidative stress. The lack of increase in GSH could be due to the utilization of the newly synthesized GSH, besides as an antioxidant, in PC<sub>s</sub> synthesis as already outlined (Cobbett, 2000). Heavy metals activate the PC synthase activity thus inducing the PC<sub>s</sub> biosynthesis, resulting in a depletion of GSH level (Zenk, 1996). In fact, in the experiment outlined before, PC<sub>s</sub> amounts increased in Cd- or in Cu-grown plants. In the same plants total ascorbate (AsA + DHA) and reduced ascorbate (AsA) contents, as well as phenolic acid amounts, increased with the rise in metal concentration (Cosi, 2001). Phenolic acids are simple polyphenols. Polyphenolic structure has the ability to inhibit free radical formation and the propagation of free radical reactions through the chelation of metal ions, especially Cu and Fe, thus preventing Fenton reactions. The phenolics may also act as antioxidants by acting as hydrogen donor, leading to the formation of phenoxyl radicals. In the presence of metals, however, it is not clear to what relative



**FIGURE 25.2** Proposed interrelation between peroxidases/phenols/AsA and the NADPH/GSH/AsA cycle. APX, ascorbate peroxidase; AsA, reduced ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbatereductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; PC<sub>s</sub>, phytochelatin; PhO<sup>•</sup>, phenoxyl radicals; PhOH, phenolic compounds; POX, peroxidases. (Redrawn from Sgherri, C. et al., *Physiol. Plant.*, 118, 21, 2003.)

extent radical scavenging or metal chelation might contribute to their antioxidative properties. Their effect could be likely dictated by structural features and their propensity to interact and penetrate lipid bilayers (Brown et al., 1998). A peroxidase/phenol/ascorbate system can represent an efficient detoxification mechanism of hydrogen peroxide in the vacuole (Takahama and Oniki, 1997). A cycle, that can occur in both the apoplast and in the vacuole, and where H<sub>2</sub>O<sub>2</sub> is scavenged by phenolics through a peroxidase, has been hypothesized (Sgherri et al., 2003). Phenolics are oxidized to phenoxyl radicals which can be reduced by AsA (Figure 25.2). DHA produced from AsA oxidation in the vacuole should be transported back into the cytosol to be regenerated to AsA. In *R. sativus* grown in excess of Cu or Cd, the amount of ASC produced was sufficient to replenish its amount that was reduced to form phenoxyl radicals to explain the increase in phenolic compounds (Cosi, 2001; Sgherri et al., 2003). An accumulation of phenolics, due to the induction of shikimate dehydrogenase (EC 1.1.1.25) and peroxidase (EC 1.11.1.7), was also observed in the hypocotyl of pepper grown in excess copper (Diaz et al., 2001). An interrelation between the peroxidase/phenol/ascorbate system of the vacuole and the NADPH/glutathione/ascorbate system of the cytoplasm can strengthen the removal of H<sub>2</sub>O<sub>2</sub>. This removal and the synthesis of PC<sub>s</sub> from GSH could not be ruled out and might increase the tolerance of plants to heavy metals.

### 25.3.1.2 Metal Compartmentation

Once inside the root cells, excess metals are translocated into a place where they can do the least damage to cellular metabolism. At the cellular level this involves storage in the vacuole or in the cell walls and at tissue level accumulation in the epidermal cells and in the trichomes. Trichomes apparently play a major role in storage and detoxification of metals. In *B. juncea*, Cd accumulation was found to be more than 40-fold higher in trichomes than in the total leaf (Salt et al., 1995a) and in tobacco a preferred storage of excess Cd in trichomes has also been observed (Choi et al., 2001). Trichomes accumulate other toxic metals, including Mn (Blamey et al., 1986) and Pb (Martell, 1974). Expression of a gene encoding a type 2 metallothionein (a metal binding protein) was localized in trichomes of bean plants (Foley and Singh, 1994). The distribution pattern of metals varies in the different leaf cell types and with plant species and metal. In roots and leaves of bush bean Cd ions seem to be mostly bound to pectic sites and histidyl groups of the cell wall (Leita et al., 1996), while in tomato suspension cultures and in root cell walls of *Silene cucubalus* a negligible amount of Cd was found (Inhoue et al., 1991) and no differences in cell wall binding were observed between

normal and Cd-tolerant plants (Verkleij et al., 1990). Most of Cd is also sequestered by cell wall anionic groups in roots of rice (Rascio et al., 2008) and in leaves of the aquatic macrophyte *Elodea canadensis* (Dalla Vecchia et al., 2005). Higher concentrations of Cu bound to the cell wall and uronic acid in the roots of *Sorghum sudanense* L. were speculated to be the main reason to restrain Cu translocation from roots to shoots (Wei et al., 2008).

The vacuole of root cells has been identified as the major site of sequestration for Zn, Cd, and Ni, and malate and citrate were found as relevant ligands within the vacuole (Krämer et al., 2000; Ma et al., 2005), although it is not clear how these ligands are sequestered (Haydon and Cobbett, 2007). Leaf compartmental analysis of buckwheat revealed that 80% of the Al—as Al-oxalate complex—was stored in the vacuole (Shen et al., 2002). A direct evidence of Zn vacuolar sequestration came from Zn uptake studies in Mg-ATP energized tonoplast vesicles isolated from Zn-hypertolerant and sensitive *S. vulgaris* (Verkleij et al., 1998). Zn-citrate was more effectively transported into the vacuole in the Zn tolerant ecotype although it is yet unknown which transporter is involved in the transport over the vacuolar membrane. Recently, Verkleij (2008) reported that the allocation patterns of Zn and Cd showed a preferential accumulation of these metals in the lower epidermis over storage in mesophyll cells, indicating that vacuolar compartmentation in leaves is less important. Furthermore, the allocation of metals in leaves could be explained by the effects of the metals on plant growth and leaf morphology and not as a specific result of tolerance mechanisms. Zn uptake in shoot vesicles of sensitive plants was higher than in root vesicles, suggesting that the enhanced tonoplast zinc transport, correlated with zinc tolerance in roots, is not very prominent in shoots (Chardonens, 1999). From *Schizosaccharomyces pombe* cells it is known that PC–Cd complexes (LMW complexes) are transported in the vacuole by Hmt1, an half-size ABC-type transporter (Ortiz et al., 1995) and a “high molecular weight” (HMW) complex is formed. No protein responsible for this activity has so far been found in plants, but the fact that in mesophyll cells of tobacco most of Cd and PCs were found in the vacuole (Vögeli-Lange and Wagner, 1990) and isolated in oat tonoplast vesicles displaying ATP-dependent uptake of Cd–PC complexes (Salt and Rauser, 1995), suggests that a similar process might be operational also in plant cells. Some authors observed a disappearance of PC complexes after several days and in plant samples from metal-rich sites no PCs were detected (Leopold et al., 1999). The vacuolar complex may dissociate because of the acidic vacuolar pH, and Cd might be complexed by vacuolar organic acids (citrate, oxalate, malate) (Krotz et al., 1989) and, possibly, by amino acids.

Another mechanism of vacuolar sequestration derives from studies on tonoplast-enriched vesicles of oat: a  $\text{Cd}^{2+}/\text{H}^{+}$  antiport might be involved in the accumulation of free  $\text{Cd}^{2+}$  into the vacuole (Salt and Wagner, 1993). In addition, the transporter could be a *CAX2* gene, identified as a low efficiency  $\text{Ca}^{2+}/\text{H}^{+}$  exchanger (Hirschi et al., 2000). Furthermore, tobacco plants expressing *CAX2* are able to accumulate more  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cd}^{2+}$  and *CAX2* was found to be the only vacuolar  $\text{Mn}^{2+}$ -transporter. Also nicotinamine seems to be important for sequestration of Fe in vacuoles, and it was found by immunohistochemical detection that nicotianamine (NA) concentrations increased in pea and tomato vacuoles under excess of Fe supply (Pich et al., 2001).

## 25.4 CHELATION OF THE METALS BY LIGANDS

### 25.4.1 ORGANIC ACIDS AND AMINO ACIDS

Internal detoxification of accumulated metals by chelation of heavy metal by particular ligands containing S, N, and O, may be implicated in differential metal tolerance, metal transport through xylem, and vacuolar metal sequestration. Although chelating agents can increase the translocation of metals in the xylem, they are also able to keep free metal ions within certain limits, thus reducing their toxicity. The detoxification and homeostasis of metals such as Ni and Zn is potentially correlated to their hydroxy and/or amino acids binding capacity (for a review see Rauser, 1999; Clemens, 2001), although a clear correlation between amounts of those ligands and exposure to metals has not

been presented yet. Due to their low association constant for complex formation and because high concentrations of these ligands are present in many plants independent from heavy metal tolerance, it is rather unlikely that these organic compounds are involved in tolerance mechanism.

Thanks to the improved technologies, *in vivo* molecular biology studies allowed to identify the most important long-distance Cu transporters (Herbik et al., 1996; Pich and Scholz, 1996; Liao et al., 2000; Takahashi et al., 2003; Kim et al., 2005; Irtelli et al., 2009), which seems to be all amino acid compounds. The nonproteinaceous amino acid NA plays a key role in copper complexation in xylem sap. Much of the understanding of the physiological functions of NA comes from the NA-deficient tomato mutant *chloronerva*, which contained excess copper in roots but failed to transport normal amount of copper into mature leaves. Furthermore, xylem exudates from mutant plants showed unusually low levels of copper (Herbik et al., 1996; Pich and Scholz, 1996). These results were confirmed by Liao et al. (2000) who reported that NA is likely the most important Cu ligand in tomato and chicory xylem exudates. However, the xylem transport of Cu was efficient even in the absence of NA, provided that histidine (His) was present (Liao et al., 2000). Notably, as far as the stability constant is concerned, His ( $\log K_{st} = 17.5$ ) could compete with NA ( $\log K_{st} = 18.6$ ) as a ligand for Cu. Another important enzyme in the regulation of the level of NA is the nicotianamine aminotransferase (NAAT) that catalyses the amino group transfer of NA in the biosynthetic pathway of phytosiderophores. The gene that encodes NAAT from barley was introduced into the non-graminaceous plant, tobacco. Transgenic tobacco plants (*naat* tobacco) showed a lower concentration of Cu, Mn, Fe, and Zn in both young leaves and flowers than the wild type. This lower concentration, attributable to the depletion of endogenous NA, is a further confirmation of the role of NA in metals transport (Takahashi et al., 2003). Recently, NA and His/proline were found to be the most important Cu chelators in xylem sap of *Brassica carinata* under condition of Cu deficiency and excess, respectively (Irtelli et al., 2009). The increase in NA detected in conditions of copper starvation but not in copper excess seems indicate that NA is not involved in the response to excess of copper but participates in Cu transport to the shoots in conditions of deficiency. This could be explained when the biosynthetic pattern of NA is taken into consideration. NA is an intermediate in mugineic acids (MAs) biosynthesis formed via the NA synthase-catalyzed trimerization of S-adenosyl-L-methionine. MAs are naturally secreted from graminaceous plants in iron starvation conditions in order to solubilize Fe in the soil. Unlike MAs, NA is not secreted from the roots but it is not unreasonable to think that its increase is involved in internal copper transport when plants are in metal deficiency rather than in metal excess. NA may also be important for Ni tolerance. A number of recent studies shows that transgenic overexpression of nicotianamine synthase (NAS) in *Arabidopsis* or tobacco confers increased tolerance to Ni (Douchov et al., 2005; Kim et al., 2005; Pianelli et al., 2005).

#### 25.4.2 ROOT EXUDATES

The exudation of organic molecules from the roots seems to be linked to the exclusion mechanism, and the exudation of organic acids is considered one of the most important strategies by which plants can exclude metals such as Al, Cd, and Pb by chelating them in the rhizosphere or in the apoplastic space, thus preventing their entry into the symplast (Zheng et al., 1998; Matsumoto, 2000; Yang et al. 2000; Hill et al., 2002; Watanabe and Osaki, 2002).

The efflux of organic substances is regulated by the electrochemical gradient existing across the plasma membrane (Ryan et al., 2001) due to the activity of ATP-driven proton pumps ( $H^+$ -ATPases). Organic acids typically flow across the lipid bilayer at a slow rate in response to the electrochemical gradient. However, efflux may be greatly increased under stress conditions such as toxic metal excess due to the expression (coding) of anion channels embedded in the plasma membrane or to their upregulation (Rengel, 2002). In this way, some metal-tolerant species can restrict uptake and translocation of metals maintaining low metal level in the shoots over a wide range of external concentrations (Baker, 1981). The introduction of a *Pseudomonas aeruginosa* citrate synthase (CS)

gene into tobacco and papaya, caused an increase in the CS activity in citrate exudation and in Al tolerance in transgenic plants (De la Fuente et al., 1997). High citrate, malate, and oxalate exudation from roots was found in Al-excluder plants such as Al-resistant cultivars of *P. vulgaris* (Miyasaka et al., 1991), *Paraserianthes falcataria* L. Neilson, *Acacia mangium* Wild. (Osawa et al., 1997), *Fagopyrum esculentum* (Ma et al., 1997, 2001b) and *Brachiaria brizantha* (Ishikawa et al., 2000), confirming the role of this organic acid in Al exclusion and tolerance. Current evidences suggest that efflux of malate affects the differential Al tolerance among genotypes of several crop species influencing their capacity of excluding Al from the apical tissues by chelating it in the apical rhizosphere or, more likely, in the apoplastic space. Several studies have shown that root tips of tolerant wheat genotypes exhibit an Al-stimulated efflux of malate that is minimal or absent in Al-sensitive lines (Delhaize et al., 1993; Basu et al., 1994a; Ryan et al., 1995a,b; Pellet et al., 1996). However, Parker and Pedler (1998) probing the “malate hypothesis” of differential aluminum tolerance in wheat by using other rhizotoxic ions as proxies for Al argued that malate efflux plays at most a minor role in the differential tolerance of wheat, and that a more integrative, multifaceted model of tolerance is needed. Qin et al. (2007) found that Al induced root exudation of oxalate and citrate, Cu induced exudation of oxalate, malate, and formate, and Zn induced exudation of formate from an aseptically grown poplar. However, in that study, no evidence of the organic acids involvement in heavy metals exclusion and tolerance was reported. Conversely, the release of oxalate was found to be responsible for a differential Pb tolerance among rice varieties (Yang et al., 2000). Molecules other than organic acids have been found in root exudates of plants exposed to Al. The high exudation rate of the phenolics catechol, catechin, quercetin, and curcumin, identified in three maize varieties, were found to be correlated with their different resistance to Al. However, in the same varieties the exudation of organic acids, besides being small, did not correlate with the resistance to Al shown by the three varieties (Kidd et al., 2001). Upon exposure to Ni, the nonhyperaccumulator *Thlaspi arvense* exuded histidine and citrate more than the hyperaccumulator *Thlaspi caerulescens* suggesting that the exudation of both compounds may be a strategy to exclude Ni but clearly it is not involved in Ni hyperaccumulation (Salt et al., 2000). On the contrary, in Norway spruce (*Picea abies*) (Heim et al., 2000) and in four tropical woody species (Nguyen et al., 2003) exposed to Al toxicity, the phenolic and amino acid exudates did not account for interspecific differences in Al tolerance. Cultivars of *Triticum aestivum* differing in resistance to Al were grown in the presence or absence of Al. Differences in exudation of total polypeptides, enhanced accumulation of specific polypeptides, and the greater association of Al to high molecular mass fraction from Al-resistant cultivars in response to Al stress, suggested that root exuded polypeptides may play a role in plant response to Al toxicity (Basu et al., 1994b, 1997).

In conclusion, unequivocal evidence for a function of these compounds in plant metal tolerance has been difficult to obtain.

### 25.4.3 PHYTOCHELATINS

Phytochelatins (PC<sub>n</sub>) are a family of small nonprotein metal-binding polypeptides, broadly classified as Class III metallothioneins (MT<sub>3</sub>), first sequenced by Grill et al. (1985) in *Rausolvia serpentina*. These compounds are synthesized, in the cytoplasm, from reduced glutathione (GSH) by a  $\gamma$ -glutamyl-cysteine dipeptidyl transpeptidase, called phytochelatin synthase (PCS), which is activated and post-translationally regulated by metal ions (Oven et al., 2002) and have a general structure  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$  with  $n = 2\text{--}11$ , although PC<sub>2</sub> and PC<sub>3</sub> are the most common (Cobett, 2000; Schmoger et al., 2000). The amino acid glycine (Gly) may be substituted by  $\beta$ -alanine, serine or glutamine, suggesting the existence of corresponding PC homologs, homophytochelatins (h-PC<sub>n</sub>), in different plant species. Structure and synthesis of PCs have been extensively reviewed and will not be discussed here (Steffens, 1990; Rauser, 1999; Cobett, 2000; Sanità Di Toppi et al., 2002).

There has been considerable debate concerning the functions of PC<sub>n</sub>. A wide range of metals, among which the most effective seem to be Ag, As<sup>+3</sup>, Cd, Cu, Hg, and Pb, has been seen to induce

the formation of PC<sub>s</sub>, but there is only limited evidence supporting their potential role in heavy metal tolerance (Ernst et al., 1992; Schat and Kalff, 1992; Meharg, 1994; Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000).

As yet, the only unambiguous established function of PC<sub>s</sub> seems to be Cd, Hg, and As detoxification. The evidence of this came from the characterization of the PC-synthase-deficient *Arabidopsis cad1* mutants, hypersensitive to Cd and Hg but no or hardly sensitive to any essential heavy metal micronutrient (Howden et al., 1995; Ha et al., 1999; Hall, 2002). Inhibition of PC synthesis by buthionine sulfoximine (BSO), a  $\gamma$ -glutamyl cysteine synthase ( $\gamma$ -ECS) inhibitor, enhanced Hg sensitivity in *Vallisneria spiralis* and *Hydrilla verticillata* (Gupta et al., 1998) and BSO-treated cell cultures of tobacco and *Rauvolfia serpentina* showed hypersensitivity to As (Nakazawa et al., 2000; Schmoger et al., 2000). Disruption of the PCS gene in *S. pombe* resulted in hypersensitivity to Cd (Clemens et al., 1999; Ha et al., 1999). In *Saccharomyces cerevisiae* the expression of PCS cDNA from wheat, *Arabidopsis*, and *S. pombe* increased Cd tolerance and Cd-hypersensitive *Arabidopsis* mutants were impaired in PC synthesis (Howden et al., 1995; Cobbett et al., 1998). In addition, tomato cell lines selected for hypertolerance to Cd exhibited enhanced PC synthesis under Cd exposure (Chen and Goldsbrough, 1994). However, Schat and collaborators (2002) found that in nonmetallicolous and metallicolous population, the ability to induce PC accumulation decreased in the order As/Cd/Cu > Zn > Ni/Co, and was consistently higher in nonmetallicolous plants than in hypertolerant ones, except for the case of As. The sensitivities to Cu, Zn, Ni, and Co were unaffected by BSO treatments suggesting that PC-based sequestration is not essential for constitutive tolerance to these metals. Cd sensitivity was considerably increased by BSO only in nonadapted plants, whereas BSO increased As sensitivity both in nonadapted and As-hyper-tolerant plants. In conclusion, PC-based sequestration seemed to be essential only for constitutive tolerance to Cd, while it is essential for both normal constitutive tolerance and adaptative hyper-tolerance to As. Cd-treatments are also known to stimulate sulfur assimilation required for sulfide formation in plants (Nussbaum et al., 1998; Robinson, 1989). CdCl<sub>2</sub> (10  $\mu$ M) in the nutrient solution induced a 100% increase in sulfate uptake by maize roots (Nocito et al., 2002). Further evidence against direct roles of PC<sub>s</sub> for Cu or Zn tolerance has been presented. In *S. vulgaris* *in vivo* inhibition of PC synthesis by BSO decreased Cd tolerance but did not affect Zn and Cu tolerance (De Knecht et al., 1992; Schat and Vóijjs, 1997). Although Cu *in vitro* activates PC synthase and PC<sub>s</sub> can chelate Cu *in vitro*, PC<sub>s</sub> do not seem involved in Cu homeostasis *in vivo*. Higher level of PC<sub>s</sub> in tolerant plants than in sensitive ones has been reported (Steffens, 1990; Rauser, 1999), and at equal external Cu concentrations PC production was significantly higher in the metalliferous clones than in nonmetalliferous clones of *H. lanatus*, *S. vulgaris*, and *S. cucubalus* (Schat and Kalff, 1992; De Vos et al., 1992; Hartley-Whitaker et al., 2001b). However, the comparison of PC production at Cu NEC or EC<sub>50</sub> revealed that PC<sub>s</sub> were equal at the same level of stress. Therefore, it has been concluded that PC<sub>s</sub> are not decisively involved in differential copper tolerance in *S. vulgaris*, *S. cucubalus*, and *H. lanatus* (Schat and Kalff, 1992; De Vos et al., 1992; Hartley-Whitaker, 2001a). In addition, Cu hypertolerance is associated with strongly decreased PC synthesis under Cu exposure (De Vos et al., 1992). More recently it has been shown that in *Escherichia coli* or *S. cerevisiae* Cd tolerance of cells increases with higher levels of expression of PCS gene (*AtPCSI*) (Matsumoto et al., 2004); however, the over expression of this gene does not lead to increased Cd tolerance and accumulation in *Arabidopsis thaliana* (Lee et al., 2003). Although suppression of the high-affinity phosphate uptake system is doubtlessly the major genetic determinant of As (V) hypertolerance (Meharg and MacNair, 1992; Meharg, 1994; Wang et al., 2002; Bleeker et al., 2006), it cannot be excluded that the precise level of tolerance is influenced by other additional determinants besides the rate of As(V) uptake. Schmöger et al. (2000) have clearly demonstrated the formation of PC-As complexes *in vitro* and *in vivo*, but indications for a potential role of PC<sub>s</sub> in As hypertolerance came from studies which demonstrated that PCS-deficient *A. thaliana* showed hypersensitivity to As(V) (Ha et al., 1999), and that in hypertolerant *H. lanatus*, which exhibited elevated PC-thiol: As ratios, BSO abolished As tolerance (Hartley-Whitaker et al., 2001b), indicating that PC<sub>s</sub> are essential for As(V). The first step in As(V) detoxification could be

the reduction to As(III) catalyzed by the plant arsenate-reductase (Bleeker et al., 2006). Several studies have suggested the reduction of arsenate to arsenite by endogenous arsenate reductase in plant cells (Pickering et al., 2000; Dhankher et al., 2002). Bleeker et al. (2006) demonstrated that an enhanced arsenate reductase activity improves arsenate internal detoxification, promoting the formation of As(III)-GS<sub>3</sub> or As(III)-PC complexes (Raab et al., 2004, 2005), which are then transported in the vacuole, likely by an ABC-type transporter. Recently, the presence of an arsenate reductase activity from a root extract of *P. vittata* that reduces arsenate to arsenite in *in vitro* assays has been reported (Duan et al., 2005) and a gene encoding a putative endogenous arsenate reductase from *A. thaliana* that reduces As(V) to As(III) has been identified in plants (Dhankher et al., 2006). However, the As hyperaccumulator fern, *P. vittata*, which accumulates As as As(III) produced only very low amounts of PC<sub>s</sub> upon exposure to As(V) (Zhao et al., 2003). However, little is known about As tolerance and the detoxification mechanism in this hyperaccumulator. More recently it was shown that arsenate tolerance in *Silene paradoxa*, from a mine site enriched in As and in other heavy metals, did not rely on arsenate reduction and subsequent PC-based sequestration (Arnetoli et al., 2008). Schulz et al. (2008) instead found that the toxicity of As(V) in six plant species with different As sensitivity under sufficient phosphate nutrition is dependent on PCs production but arsenic tolerance is not associated with an extended biosynthesis of long-chain PC<sub>s</sub>. Generally, in As-tolerant plant species, PC<sub>s</sub> with a shorter chain length dominate, PC2 being the dominant phytochelatin. With increased As sensitivity, the production of PC3 and PC4 are increased.

The previous authors suggest that the PC2/PC3/PC4 ratio in roots of those plants could be useful for early recognition of arsenic exposure and as a tool to assess the degree of As sensitivity. Further studies are, therefore, required to unequivocally understand the mechanism involved in As(III) and As(V) tolerance.

Recently, it has been reported that soybean plants showed a notably high contribution of homogluthathione (hGSH)—a compound which in some legumes replaces GSH synthesizing homo-phytochelatins (h-PC<sub>s</sub>) instead of PC<sub>s</sub>—to the pool of thiols in shoots under both Cd and arsenate exposure. However, a higher level of hPC<sub>s</sub> in Cd-treated soybeans compared to PC<sub>s</sub> in lupins did not prevent growth inhibition. In contrast, the highest thiol concentrations in soybean exposed to arsenate were associated with reduced growth inhibition of roots; therefore, the role of hPC<sub>s</sub> in the arsenate detoxification mechanisms of this species seems to be clearer, showing higher thiol concentrations and lower growth reduction than those present in lupin plants (Vázquez et al., 2009).

In conclusion, an overproduction of PC<sub>s</sub> in plants exposed to heavy metals does not seem a general likely mechanism for metal tolerance, owing to the energy required for sulfate reduction to support PC synthesis (Steffen, 1990).

Aside from detoxification, PC<sub>s</sub> are considered to have an important role in cellular essential metal-ion homeostasis (Steffen, 1990). Some metal ions, such as Zn<sup>2+</sup> and Cu<sup>2+</sup> are part of catalytic proteins or structural elements to allow for metabolism in plants. While there is, as yet, no direct evidence that PC<sub>s</sub> play a role in essential metal homeostasis, *in vitro* experiments have shown that PC–Cu and PC–Zn complexes could reactivate the apo forms of the Cu-dependent enzyme diamino oxidase and the Zn-dependent enzyme carbonic anhydrase, respectively (Thumann et al., 1991). Thus, the production of PC<sub>s</sub> and of metal-PC complexes may be seen as a first and transient response of the cells since their production generally decreases or even disappears with duration of time of heavy metals exposure (Leopold et al., 1999; Wójcik and Tukiendorf, 2003). PC complexes have been also identified as a possible transport form of Cu from roots to leaves in Cu-exposed *Larrea tridentata* (Polette et al., 2000). Therefore, according to other recent studies further roles have been proposed for PCs. It is conceivable that PC<sub>s</sub> may also be involved in the long-distance transport of Cd, and possibly other heavy metals. In transgenic *A. thaliana* and in grafted *Arabidopsis* plants it has been shown that PC<sub>s</sub> undergo long-distance transport in the root-to-shoot and shoot-to-root directions (Gong et al., 2003; Chen et al., 2006), and that in addition to the directional xylem transport, the phloem results a major vascular system for long-distance source to sink transport of Cd as



PC-Cd and glutathione-Cd complexes (Mendoza-Cózatl et al., 2008). However, future researches are needed to understand the mechanism by which thiol, Cd and/or thiol-Cd complexes are loaded and unloaded from the phloem.

#### 25.4.4 METALLOTHIONEINS

Metallothioneins (MT<sub>s</sub>) are small protein (MW 6–8 kDa) cysteine (Cys) rich proteins that can bind metals via the thiol groups of their Cys residues. The first demonstration that plants could produce, in addition to PC<sub>s</sub>, these metal-binding proteins comes from the purification of the wheat embryo Ec protein, the first MT purified from a plant tissue, and characterized as a Zn-binding protein involved in the regulation of Zn homeostasis during early seed germination (Lane et al., 1987). Since this discovery, Mt genes have been found throughout the plant kingdom (Cobbett and Goldsbrough, 2002). MT<sub>s</sub> have been divided into two classes based on amino acid sequence. Class I includes mammalian MT<sub>s</sub>, Class II includes Mt<sub>s</sub> from plants and fungi as well as invertebrate animals. Plant MT<sub>s</sub> are further classified into four types according to the arrangement of Cys residues (Cobbett and Goldsbrough, 2002). Based on the analysis of MT RNA expression in a number of plant species, type 1 Mt genes are, in general, expressed more in roots than in shoots, whereas type 2 Mt genes are expressed primarily in leaves (Hsieh et al., 1995, 1996). Type 3 genes are expressed in leaves or in ripening fruits (Ledger and Gardner, 1994), while expression of type 4 MT<sub>s</sub> appears to be restricted to developing seeds (White and Rivin, 1995). For the genome of *A. thaliana* seven putative MT genes have been annotated (plus one pseudogene), including the previously characterized genes AtMT1, AtMt2a/2b, and AtMT3 (Guo et al., 2003). The diverse patterns of expression of different MT genes suggest that plant MT isoforms may differ not only in their amino acid sequence but also in the function they perform in specific tissues. However, expression studies have revealed that different MT isoforms exhibit overlapping expression patterns, pointing to partial function redundancy (Guo et al., 2003).

In the aim of enhancing metal tolerance, sequestration, or accumulation of plants, the high metal-binding capacity of MT<sub>s</sub> has been widely exploited.

Plant MT1 and MT genes have been shown to complement MT-deficient yeast (cup 1 D), demonstrating their function in copper detoxification (Zhou and Goldsbrough, 1994). Expression of plant MT1 and MT2 in the yeast mutant cup 1 D also increased cadmium tolerance. Likewise, expression of plant MT<sub>s</sub> in *E. coli* led to increased tolerance toward copper and cadmium (Evans et al., 1992; Zhigang et al., 2006). Based on these findings, plant MT<sub>s</sub> are thought to play a major role in cellular copper and cadmium homeostasis. However, when MT2 gene from *A. thaliana* was expressed in a *Synechococcus* mutant deficient in its Zn-metallothionein gene *smtA*, a partial complementation was achieved, pointing to a possible role for MT2 in plant zinc homeostasis (Robinson et al., 1996).

Only few studies have exploited the *in vivo* functions of MTs in plants. Progresses were hampered by the difficulties in detecting these proteins because of their instability and tendency to oxidize under normal protein isolation conditions (Murphy et al., 1997). In *E. coli* and *A. thaliana*, over expressing a MT-like gene from pea, PsMT<sub>A</sub>, an up to eight-fold accumulation of copper was reported (Evans et al., 1992). MT2a and MT3 of *A. thaliana* were transiently expressed in *Vicia faba* guard cells (Lee et al., 2004), which showed an enhanced resistance to cadmium exposure. A MT2 protein from *B. juncea*, BjMT2, was over expressed in *A. thaliana* under the control of the 35S promoter. The transformed seedlings exhibited an increased tolerance to copper and cadmium on the basis of shoot growth and chlorophyll content. Analysis of transiently transformed cells of *A. thaliana* leaves revealed exclusive cytosolic localization of BjMT2:EGFP (Enhanced Green Fluorescent Protein) fusion protein in control and heavy metal-exposed plant cells. Remarkable, ectopic expression of BjMT2 reduced root growth in the absence of heavy metal exposure, whereas in the presence of copper root growth in control and transgenic lines was the same indicating that in *A. thaliana*, root and shoot development are differentially affected by ectopic expression of BjMT2 (Zhigang et al., 2006).

Although MT2 mRNA<sub>s</sub> increase in response to a great variety of stresses, MT2 gene expression seems to be required for tolerance to heavy metals, especially copper (Rausser, 1999). MT2 expression was

the primary determinant of ectopic differences in the copper tolerance of *Arabidopsis* seedlings (Murphy and Taiz, 1995) and MT expression was induced by Cu and, though less effectively, by Cd and Zn (Zhou and Goldsbrough, 1995). However, in some plant species no induction of MT2 transcript levels was found after copper exposure (Schafer et al., 1997; Thomas et al., 2003).

A study on *S. vulgaris* showed that in copper-tolerant ecotypes, a MT gene was much more expressed (SvMT2b) than in the sensitive ecotypes, independent of Cu exposure (Van Hoof et al., 2001). This constitutive MT expression contrasts with an inducible MT2b expression in *A. thaliana* and points out that Cu tolerance mechanism is fundamentally different from Cu detoxification (Verkleji, 2008). A similar mechanism was observed in *S. paradoxa*. The levels of copper tolerance and constitutive MT2b expression were increased in cupricolous populations as compared to the serpentine and nonmetallicolous populations (Mengoni et al., 2003). The results of the latter author, together with those of Van Hoof et al. (2001) allow to argue that the MT2b locus represents a major target for natural selection imposed by soil copper toxicity. However, the over-expression of SvMT2b did not produce Cu tolerance per se but merely enhanced the degree of hypertolerance in already tolerant plants. Therefore, MT2b seems to act as a hypostatic enhancer, rather than as a primary tolerance gene (Van Hoof et al., 2001).

Although to date, the relationship of plant MTs in metal detoxification has been widely studied only for copper and cadmium, there is a growing body of evidence that these cys-rich proteins might be involved in the tolerance of other metals. Arsenic is able to induce MT synthesis in mice and humans (Liu et al., 2000; Garret et al., 2001) and the As binding to mammalian MTs has been demonstrated as well (Toyama et al., 2002; Jiang et al., 2003). However, the role of plant MTs in As binding and detoxification is still unexplored. Trivalent As bound to MTs from *Fucus vesiculosus* has been reported and five arsenic-MT complexes with increasing As to protein ratio have been characterized (Merrifield et al., 2004). In addition, it has been demonstrated that *A. thaliana* MT1 proteins were stabilized by metals among which As (Zimeri et al., 2005). Zhang et al. (2005), studying the heavy metal tolerance mechanism of *Allium sativum* L., firstly demonstrated that the transcript level of AsMT2a in roots was significantly increased by Na<sub>3</sub>AsO<sub>4</sub>. Recently, preliminary details of three *Prosopis juliflora* Mt genes have been provided (Usha et al., 2009). All three PjMTs demonstrate the ability to bind Cd, Zn, and Cu with PjMT1 showing maximum binding to the three heavy metals. PjMT1 and PjMT2 seems to be involved in copper and zinc homeostasis, respectively, while PjMT3 might be involved in heavy metal detoxification.

In conclusion, many decades of intensive reports have addressed MT structure, functions, and gene expression, but despite the increasing experimental data, several topics remain to be clarified, and the true function of this elusive protein (Palmiter, 1998) has yet to be disclosed.

## 25.5 MYCORRHIZAS AND HEAVY METAL TOLERANCE

Mycorrhizal fungi are a major component of the rhizosphere, where they establish mutual association with the roots of most plant species. Numerous studies provided evidence that these fungal symbionts can be effective in the heavy metal tolerance by helping the host plant to cope with the metal toxicity, therefore, root mycorrhization can be seen as one of the means that allow metallophytes to thrive on metal polluted soils (Hildebrandt et al., 2007; Rodriguez and Redman, 2008). The highlighting of the ameliorating effects of root-fungus associations on plant heavy metal tolerance strengthened the interest in mycorrhizal fungi and in understanding the mechanisms whereby they can alleviate the heavy metal stress in the host plant; this in view of the potential use of plant mycorrhization in ecological reclamation of contaminated soils and in revegetation of mine tailing that usually can hardly support any plant growth (Göhre and Paszkowski, 2006).

Large diversities in metal response and metal specificity have been found among different mycorrhizal fungi; therefore, the ameliorating effects strongly depend on the fungal species or genotype. The most efficient in plant protection turn out to be the fungal isolates from methallicolous soils, which are more adapted to the heavy metals (Adriaensen et al., 2003).

As regards ectomycorrhizas, common among forest trees, it has been shown, for instance, that Zn or Cu tolerant isolates of *Suillus bovinus* provide excellent insurance against these heavy metals in Scot pine (*Pinus sylvestris*) seedlings (Van Tichelen et al., 2001; Adriaensen et al., 2003) and that specific genotypes of *Suillus luteus* are very efficient in protecting pines from Cu (Adriaensen et al., 2005) or Cd (Krzmaric et al., 2009). Most mechanisms proposed for ectomycorrhizas to explain their ameliorating effects involve exclusion processes that restrict metal movement to the host roots. The tolerant fungi retain the heavy metals by means of strategies similar to those employed by higher plants, namely through extracellular sequestration (binding them to slimes, cell walls or extruded ligands) and intracellular detoxification (storing them in the vacuolar compartment or complexing to chelators such as GSH and metallothioneins) (Bellion et al., 2006). Indeed, the Cu and Zn tolerance of ectomycorrhizal fungus *Pisolithus tinctorius* relies on the heavy metal absorption by extrahyphal slime (Tam, 1995) whereas in *Paxillus involutus* Cd is both bound to cell walls and stored in vacuoles (Blauzer et al., 2000), and Cd and Cu are also sequestered in the cytosol as metallothionein complexes (Bellion et al., 2007). Interestingly, Cd-tolerant *Suillus-Pinus* and *Paxillus-Pinus* mycorrhizal associations show an enhanced symbiosis defense system with high GSH concentrations (Schützenbühl and Polle, 2002). Similarly, the Cd exposure strengthens the antioxidative system of *Paxillus involutus* with a rise of SOD- and GSH-related enzyme activities (Ott et al., 2002). Thus, it seems that ectomycorrhizal fungi can also provide the host plant with protection by “arming” the roots with physiological defenses against the heavy metal oxidative stress (Bellion et al., 2006).

The endomycorrhizal arbuscular fungi (AMFs), which are the most widespread among the mycorrhizal fungi, have been commonly reported in metal contaminated soils (Göhre and Paszkowski, 2006). Recent studies give strong evidence that AMFs can filter out toxic heavy metals by keeping them away from the roots (Hildebrandt et al., 2007) and that they can help the host plant to overcome the difficulties in acquisition of P and other mineral nutrients (Dong et al., 2008). Lin et al. (2007) reported that colonization by the AMF *Glomus mosseae* positively affected growth and Cd/Zn tolerance of three leguminous plants (*Sesbania rostrata*, *Sesbania cannabina*, *Medicago sativa*) through mycorrhizal immobilization of heavy metals and decreased translocation to the shoot. Moreover, the mycorrhization stimulated the root nodule formation, thus leading to increased N<sub>2</sub> fixation. *Glomus mosseae* was found to reinforce Cu, Cd, and As tolerance in other plants (*Trifolium repens*, *Lolium perenne*, *Coreopsis drummondii*, and *P. vittata*) as well as to improve the P nutrition and to curtail the shoot amount of toxic elements (Chen et al., 2007). Dong et al. (2008) showed that *Trifolium repens* and *Lolium perenne* heavily depended on mycorrhizas for surviving the As toxicity in an arsenic contaminated soil. In *Medicago truncatula* (Xu et al., 2008) and in barley (Christophersen et al., 2009) AMFs enhanced the As tolerance by restricting the root As uptake through the suppression of high affinity arsenate/phosphate transporters and the upregulation of the phosphate uptake system responsible for transfer of Pi from the symbiotic interface to cortical cells. Recently, genes responsive to heavy metals (Cu, Cd or Zn) exposure were studied in the AMF *Glomus intraradices* mycelium and in *Medicago truncatula* mycorrhizal roots. The finding that genes encoding proteins (GSH S-transferase and HSP90) which potentially counteract the ROS damage are upregulated by heavy metals in mycorrhizal roots seems to indicate that a primary concern of the fungal partner in the symbiosis is the heavy metal oxidative stress (Hildebrandt et al., 2007).

## 25.6 HEAVY METAL HYPERACCUMULATION

### 25.6.1 HYPERACCUMULATOR PLANTS

High tolerance to a range of heavy metals has evolved in many species exposed to elevated metal concentrations in native soils.

Although the majority of these heavy metal-tolerant plants behaves as “excluders,” a series of tolerant plants exists, which deal with heavy metals in just an opposite way. This kind of plants,

defined “hyperaccumulators” (Brooks et al., 1977), actively take up the potentially toxic metals, which are then translocated from root to shoot and accumulated in the aboveground tissues where they reach exceptional concentrations of several percent of dry weight (usually 100–1000-fold higher than those found in normal plant species), without giving rise to phytotoxic effects (Rascio, 1977; Reeves, 2006). Although a number of hypotheses have been proposed (Boyd and Martens, 1992), the evolutionary reason for metal hyperaccumulation still remains unclear. One of the most attractive proposals is that a huge concentration of toxic elements can provide the plant with an “elemental defense” against pathogens and herbivores (Boyd, 2007).

Tolerance and hyperaccumulation are two distinct features; however, the hyperaccumulators also have the characteristic of tolerance (or hypertolerance) which, according to Chaney et al. (1997), is the key property that makes the hyperaccumulation possible.

The first discovery of a plant with such an extreme ability to accumulate heavy metals goes back to 1948, when Minguzzi and Vergnano found 10,000  $\mu\text{g Ni g}^{-1}$  of dry weight in shoots of *Alisum bertolonii* from serpentine soils of Italy.

Besides the ecological and physiological interest, hyperaccumulator plants have attracted considerable attention for practical applications, because of their potential utilization in phytoremediation of metal contaminated soils (Pilon-Smits, 2005) as well as in phytomining (Li et al., 2003). Recently, the possibility of exploiting the accumulation traits of these plants for strategies of food crop biofortification has also sparked great interest (Palmgren et al., 2008). The better understanding of hyperaccumulation mechanisms, in fact, would assist the development of genetically engineered plants with improved nutritional value for the world’s populations suffering from mineral element deficiency (especially Zn) in their vegetarian diet (Maret and Sandstead, 2006).

Over 450 species of plants, belonging to a wide range of unrelated families, have been identified as hyperaccumulators of heavy metals (Ni, Zn, Cd, Cu, Co, Mn), and also of metalloids (As, Se). Most of them are endemic to metalliferous soils, but some species, classified as facultative metallophytes, although preferring metal-rich soils, can also grow on nonmetalliferous ones (Assunção et al., 2003b). Hyperaccumulators are generally minor vegetation components in most European and North American habitats, but they are relatively abundant in some locations from New Caledonia, Cuba, and South Africa (Boyd, 2004).

The majority of taxa (nearly 400) hyperaccumulate Ni, whereas there are fewer hyperaccumulators of other metals, such as Zn, Pb, Cu, and Co, and only four species hyperaccumulate Cd (Reeves, 2006; Verbruggen et al., 2009). There are also rather few plants that hyperaccumulate As (Wang et al., 2007) and Se (Terry et al., 2000).

*Sebertia acuminata* (Sapotaceae), a rare rainforest tree, endemic to the New Caledonia ultramafic serpentine soils, is the most extreme example of a hyperaccumulator. Its latex concentrates the highest Ni quantities found in an organism (up to 26% of dry mass) and a single tree may contain 37 kg of the metal (Jaffré et al., 1976; Sagner et al., 1998).

The common definition of hyperaccumulator plants meets the requirement that the metal concentration in the shoot must be higher (as % of dry weight) than a threshold, which is different for each metal, depending on its phytotoxicity. According to such a criterion, hyperaccumulators are plants that accumulate  $>10,000 \mu\text{g g}^{-1}$  (1%) Zn or Mn,  $>1,000 \mu\text{g g}^{-1}$  (0.1%) Ni, Pb, Cu, Cr, Co, and also As or Se, and  $>100 \mu\text{g g}^{-1}$  (0.01%) Cd, when grown on native soils (Baker and Brooks, 1989; Terry et al., 2000; Wang et al., 2006).

Other requirements proposed for classifying a hyperaccumulator are that the metal concentration in plant grown on heavy metal-rich soils must be 10–500 times higher than in the same plant species from non-polluted environments (Yanqun et al., 2005) and that the ratios of both shoot to root and shoot to soil metal concentration must be  $>1$  (McGrath and Zhao, 2003; Yanqun et al., 2005). However, these last criteria have recently been debated, since they may exclude rare and strictly metallophyte plants which cannot be compared with plants from unpolluted environments as well as species able to accumulate very high quantities of earth abundant metals such as Al or Fe in aboveground tissues (Branquinho et al., 2007).

The Brassicaceae family, and particularly the *Thlaspi* and *Alyssum* genera, are rich in hyperaccumulator species (approximately 25% of the documented taxa). The greatest number of Ni hyperaccumulators (about 50 taxa) belongs to the genus *Alyssum* (Brooks, 1998). The genus *Thlaspi* also includes a high number of Ni hyperaccumulators and species have also been found in genera of different other families (Baker and Brooks, 1989).

The list of Zn hyperaccumulators is shorter (<20 species) than that of the Ni ones. Most species belong to the genus *Thlaspi*, and *Arabidopsis halleri*, (Brassicaceae) (Baker and Brooks, 1989) and *Sedum alfredii* (Crassulaceae) (Yang et al., 2004) are Zn hyperaccumulators too.

The Zn hyperaccumulation in Brassicaceae is a constitutive trait at the species level. Studies on *A. halleri* and *Thlaspi caerulescens*, which are the two best known hyperaccumulators, showed that all populations can accumulate Zn, but the degree of hyperaccumulation and hypertolerance exhibits considerable variation, reaching the highest values in those from metalliferous soils (Bert et al., 2000; Assunção et al., 2003a). Certain *T. caerulescens* ecotypes accumulate Zn at levels as high as 30,000  $\mu\text{g g}^{-1}$  (shoot dry weight) when grown on nutrient solutions (Brown et al., 1995). Differently, in *S. alfredii*, the Zn hyperaccumulation is not constitutive at the species level, being confined to metallicolous populations, where Zn concentrations can reach over 20,000  $\mu\text{g g}^{-1}$  (shoot dry weight) without any symptoms of toxicity (Yang et al., 2006a; Deng et al., 2007).

Cd hyperaccumulation has only been reported in four species of Cd/Zn hyperaccumulators: *T. caerulescens* (Brown et al., 1995), *A. halleri* (Küpper et al., 2000), *S. alfredii* (Yang et al., 2004), and *Thlaspi praecox* (Vogel-Mikuš et al., 2005). In all of these, the Cd hyperaccumulation is not constitutive at the species level, only occurring in metallicolous populations which also show a considerable variation in their ability to accumulate this heavy metal (Assunção et al., 2003a; Bert et al., 2003; Roosens et al., 2003; Deng et al., 2007). The highest known capacity of Cd hyperaccumulation is that found in *T. caerulescens* populations of the “Ganges” ecotype (originally called “French A”) from Zn/Cd-rich soils of southern France, which can accumulate in the shoots >10,000  $\mu\text{g g}^{-1}$  (dry weight) without suffering phytotoxicity (Lombi et al., 2000).

In 2001, the fern *P. vittata* was identified as the first plant able to hyperaccumulate arsenic in its fronds (up to 2.3% of dry weight) (Ma et al., 2001a) and then it was demonstrated that the As tolerance of *P. vittata* results from both constitutive and adaptative traits and that this species is also able to constitutively accumulate Zn and Pb (Zhao et al., 2002a; Wu et al., 2009). After *P. vittata* other *Pteris* taxa were found to hyperaccumulate over 1,000  $\mu\text{g As g}^{-1}$  (dry weight) in fronds under field conditions (Wang et al., 2006, 2007).

Selenium hyperaccumulation has been observed in plants of different families, among which species belonging to the genus *Astragalus*, such as *Astragalus bisulcatus* (Fabaceae), and in *Stanleria pinnata* (Brassicaceae), which can accumulate Se in a range of 2,000–16,000  $\mu\text{g g}^{-1}$  of shoot dry weight (Terry et al., 2000; Galeas et al., 2007).

In hyperaccumulating plants the concentration of exceptionally high quantities of heavy metals (or metalloids) in their aboveground tissues resisting their toxic effects depends on three basic hallmarks that distinguish these plants from the related nonhyperaccumulating nontolerant species:

- A much greater ability to take up metals from the soil
- A much more rapid and efficient root to shoot translocation of the absorbed metals
- A much greater capability to detoxify and to store high metal amounts in the leaf cells

Physiological as well as genomic and transcriptomic studies carried out by comparing hyperaccumulating with related nonhyperaccumulating plants have paved the way for the understanding of metal hyperaccumulation mechanisms at functional and molecular levels.

Most studies focused on Zn, Ni, and Cd hyperaccumulation having *T. caerulescens* and *A. halleri* as model plant systems. However, analyses performed on other heavy metal or metalloid hyperaccumulators also provided useful information on the hyperaccumulation strategies. Comparative transcriptomic studies revealed that most genes thought to be involved in hyperaccumulation and

hypertolerance key steps are not novel but rather common to hyperaccumulating and nonhyperaccumulating plants, being only differently expressed and regulated in the two kinds of plants. In particular, it was found that a large gene array shows a constitutive overexpression in hyperaccumulators (Verbruggen et al., 2009).

### 25.6.2 ENHANCED HEAVY METAL UPTAKE

In roots of *T. caerulescens* and *A. halleri* a key role in enhanced Zn uptake seems to be played by the overexpression of genes belonging to the ZIP (Zinc-regulated transporter Iron-regulated transporter Proteins) family, which encodes putative plasma membrane-located cation transporters common to plants and animals (Grotz et al., 1998). In roots of *T. arvense*, like in the other nonhyperaccumulating plants, these genes are all expressed at a detectable level only under zinc deficiency, being strongly downregulated at normal Zn supply. By contrast, in *T. caerulescens* roots the expression of two ZIP genes (*ZTN1* and *ZTN2*) is barely Zn-responsive and still persists at sufficient Zn nutrition, suggesting that this high expression, irrespective of Zn availability, is the major reason for the enhanced Zn uptake in this hyperaccumulator plant (Pence et al., 2000; Assunção et al., 2001). Similarly, the lack in *A. halleri* roots of a Zn-dependent transcriptional regulation of *ZIP6* and a barely Zn-regulated expression of *ZIP9*, which are both strongly Zn-responsive in nonhyperaccumulator *A. thaliana*, lead to regard these genes as candidates for a role in Zn hyperaccumulation (Weber et al., 2004; Filatov et al., 2006). Other overexpressed but Zn-regulated ZIP genes have been identified in roots and shoots of *T. caerulescens* and *A. halleri*. Nevertheless, their involvement in Zn hyperaccumulation is not clearly established (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006).

Despite the chemical similarity between Zn and Cd, physiological evidence exists that the mechanisms of Cd uptake may be different from those of Zn uptake in roots of the Zn/Cd hyperaccumulator *T. caerulescens* and that multiple absorption systems operate in a specific way in ecotypes with different Cd hyperaccumulation capacity (Lombi et al., 2001; Zhao et al., 2002b). In the lower Cd-accumulating Prayon ecotype (from Belgium) the Zn and Cd influxes depend on a system with a strong preference for Zn over Cd. The Cd uptake significantly decreases with the increase of Zn concentration, suggesting that the Cd absorption is largely mediated by Zn transporters (Zhao et al., 2002b). Plausibly this Cd uptake involves *ZTN1* which has been shown to mediate a high affinity Zn and low affinity Cd transport (Pence et al., 2000). Moreover, the inhibition of Cd absorption by increasing Ca concentrations suggests that in this *T. caerulescens* accession the Cd uptake might also occur via Ca channels (Zhao et al., 2002b). Differently, in the higher Cd hyperaccumulator Ganges ecotype (from southern France) surviving concentrations of more than  $3,000 \mu\text{g Cd g}^{-1}$  (dry weight) in the aboveground tissues (Lombi et al., 2000), the Cd uptake occurs with the same  $K_m$  but a  $V_{max}$  fivefold higher than the Prayon one. Furthermore, it is not inhibited by Zn or other metals nor by increasing Ca concentrations. This strongly suggests the existence of a highly selective Cd transport system in root cell membranes of this high Cd accumulating accession of *T. caerulescens* (Lombi et al., 2001; Zhao et al., 2002b; Assunção et al., 2008). Interestingly, the existence of a specific and highly efficient Cd transport system, coupled with the Cd-increased plant growth (Roosens et al., 2003), raised the question as to whether Cd might play any physiological role in the high accumulating Ganges ecotype, as it occurs in the marine diatom *Thalassiosira weissflogii* where a Cd-requiring carbonic anhydrase exists (Lane et al., 2005). In a recent study, Liu et al. (2008) provide evidence of a positive correlation between carbonic anhydrase activity and Cd concentration in the shoots of Ganges plants, suggesting that Cd might actually play a physiological role in this high accumulating ecotype by enhancing the activities of some enzymes.

In the other Zn/Cd hyperaccumulator, *A. halleri*, the Cd amounts in roots and shoots dramatically decrease with the supply of high Zn concentrations, thus implying that Cd and Zn uptake is mediated by the same transport system with a higher Zn preference (Küpper et al., 2000). Besides the ability to hyperaccumulate Cd in the aboveground tissues, *A. halleri* also shows a Cd hypertolerance

due, at least in part, to a lower short-term Cd uptake rate and a very efficient sequestration of Cd ions in root cells (Bert et al., 2003; Weber et al., 2006). Finally, in *S. alfredii*, a high Zn supply enhances the Cd concentration in roots and aerial organs, suggesting the existence, in this species, of synergic Cd and Zn interactions for both absorption and transport (Yang et al., 2004).

Hitherto candidate transporters mediating the Ni uptake in the hyperaccumulating plants have not been identified (Verbruggen et al., 2009). Since several serpentine populations of Zn/Ni hyperaccumulators prefer Zn over Ni under equimolecular supply of the two metals (Assunção et al., 2001, 2008) the involvement of a Zn transport system in Ni uptake has been supposed. However, the finding that at low Ni concentrations the rates of Ni uptake and root-to-shoot translocation are similar in both the Ni-hyperaccumulator *Thlaspi goesingense* and the non-Ni-hyperaccumulator *T. arvense*, led Krämer et al. (1997) to suggest that the Ni hyperaccumulator phenotype relies primarily on a remarkable Ni tolerance achieved through efficient leaf systems of metal detoxification rather than on the enhanced heavy metal uptake/translocation.

As regards the arsenic hyperaccumulation, ample evidence exists that plants take it up as arsenate via the transport system of the chemically analog phosphate (Meharg and Hartley-Whitaker, 2002) and that the hyperaccumulator fern *P. vittata* is much more efficient in arsenate uptake than other nonhyperaccumulating species (Caille et al., 2005; Lou et al., 2009). This enhanced As absorption seems to result from a greater density of phosphate/arsenate transporters due to the gene overexpression in the root cells (Caille et al., 2005). Furthermore, the lower  $K_m$  of *P. vittata* compared with the nonhyperaccumulating fern *Nephrolepis exaltata*, shows a higher arsenate affinity of the hyperaccumulator transport proteins (Poynton et al., 2004).

In Se hyperaccumulating plants, such as *A. bisulcatus*, and *Stanleya pinnata*, the selenium uptake, mainly as selenate, occurs through sulfate transporters, due to the chemical similarity between selenium and sulfur (Terry et al., 2000). More precisely, the selenate absorption involves specifically high-affinity sulfate transporters localized exclusively in the root cells (Shibagaki et al., 2002; Sors et al., 2005b). In nonhyperaccumulating species transporters of this kind are inducible and regulated by the S status of the plant (Hirai et al., 2003). The finding in hyperaccumulator plants of Se/S ratios 100-fold higher than in related nonhyperaccumulating species strongly suggests that one or more sulfate transporters may have evolved into Se-specific transporters unresponsive to the plant S status (Sors et al., 2005b; Galeas et al., 2007).

### 25.6.3 IMPROVED ROOT TO SHOOT HEAVY METAL TRANSLOCATION

A common trait of heavy metal (or metalloid) hyperaccumulation, after the uptake from the soil into the root, is the fast and efficient translocation from root to shoot of the absorbed heavy metals. This is a key step requiring a symplastic radial flow followed by an active loading into the xylem vessels. The enhanced translocation to the shoot entails a limited sequestration into and a fast release from the root cell vacuoles to make higher metal amounts readily available for the xylem loading. This could indicate differences in root tonoplast characteristics accounting for a smaller influx and a larger efflux of metals (Lasat et al., 2000). It has been found, indeed, that 2.7-fold less Zn is stored in root vacuoles of the hyperaccumulator *S. alfredii* compared with the nonhyperaccumulating ecotype (Yang et al., 2006b). A much lower Zn amount (2.5-fold) is also sequestered in root vacuoles of *T. caerulescens* when compared to *T. arvense* and, furthermore, the rate of Zn efflux out of the vacuoles is about twofold faster in the hyperaccumulator plant (Lasat et al., 2000). Recently, Xing et al. (2008) reported an inverse relation between Cd storage in root vacuoles and translocation efficiency in *T. caerulescens* accessions differently able to accumulate Cd.

In enhanced xylem loading of hyperaccumulators, the key role is played by some types of transporters among which  $P_{1B}$ -type ATPases, also known as heavy metal transporting ATPases (HMAs), a class of proteins mediating the heavy metal ion transport in plants, with roles in homeostasis and metal tolerance (Axelsen and Palmgren, 1998). The expression of the *HMA3* and *HMA4* genes, encoding two HMAs of the bivalent cation transporter group, is significantly higher in both *T. caerulescens*

and *A. halleri* than in *A. thaliana*, with transcript levels 2–3-fold higher in roots than in shoots of the two hyperaccumulators (Papoyan and Kochian, 2004; Talke et al., 2006; Courbot et al., 2007). Furthermore, the HMA4, which is localized at the plasma membrane of xylem parenchyma cells, where it mediates the active heavy metals efflux, is upregulated in *T. caerulescens* (Papoyan and Kochian, 2004) and, conversely, downregulated in *A. thaliana* (Mils et al., 2003) upon plant exposure to high Zn and Cd concentrations. Interestingly, in *T. caerulescens*, HMA4 is also induced by Zn deficiency, supporting the idea of a role in both heavy metal hyperaccumulation and Zn nutrition (Papoyan and Kochian, 2004). HMA4 is constitutively overexpressed also in *A. halleri*, where it possibly mediates both Zn and Cd transport from roots to shoots (Talke et al., 2006).

Another gene with a constitutive higher expression in roots of *T. caerulescens* and *A. halleri* compared to *A. thaliana* is *FRD3* encoding a member of the MATE (Multidrug And Toxin Efflux) family of small organic molecule transporters (Verbruggen et al., 2009). In *A. thaliana* *FRD3*, localized at the plasma membrane of root pericycle cells, mediates the efflux into the vascular tissue of citrate, a low molecular weight chelator necessary for root-to-shoot Fe translocation (Durrett et al., 2007). The overexpression of *FRD3* in *T. caerulescens* and *A. halleri* is supposed to contribute to Fe homeostasis and mobility in presence of higher xylem Zn concentrations requiring an improved metal chelation capacity. However, it cannot be ruled out that *FRD3* might be involved in homeostasis and translocation of other metals, including Zn (Krämer et al., 2007).

The hyperaccumulator plants exhibit constitutively elevated concentrations of organic acids, mainly citrate and malate (Montargès-Pellitier et al., 2008), which are assumed to play a role as heavy metal chelators in hyperaccumulation strategies. However, the necessity of organic acids as heavy metal ligands in roots and during the long-distance xylem transport is rather controversial. This is because of the low association constants of organic acids with metals that seem to limit their role to the metal storage in cell vacuoles, where the metal-organic acid complexation is favored by the acidic environment (Haydon and Cobbett, 2007). Sarret et al. (2002) found Zn-malate and Zn-citrate in *A. halleri* roots, whereas Zn-organic acid complexes were not detected in roots of *T. caerulescens* (Salt et al., 1999). Only 20% of the xylem sap Zn in *T. caerulescens* is complexed with citrate, the remaining 80% being present as free ion (Salt et al., 1999); in the xylem sap of *A. halleri* about half of the Zn is associated with citrate while most Cd is transported in the free ionic form (Ueno et al., 2008). Moreover, only one-third of Ni is complexed with citrate in the xylem of the Ni-hyperaccumulator *Stackhousia tryoni* (Bidwell, 2001). By contrast, Schaumlöffel et al. (2003) showed that most Ni was bound to citrate in the latex of the Ni-hyperaccumulator *S. acuminata* and Callahan et al. (2008) identified a number of other organic acids as Ni ligands in the latex of this plant.

In heavy metal hyperaccumulation and transport, a role as metal chelators is played by other small organic compounds, as free amino acids, mainly histidine (His), and NA, which are known to form stable complexes with several bivalent cations (Callahan et al., 2006). NA is present at higher concentrations in roots of both *T. caerulescens* and *A. halleri* than in those of the nonhyperaccumulators *T. arvense* and *A. thaliana* (Weber et al., 2004; Mari et al., 2006). This for the constitutive overexpression in hyperaccumulating plants of the *NAS2* gene encoding one isoform of NA synthase (Weber et al., 2004; van de Mortel et al., 2006). In *A. halleri*, the enhanced NA synthesis is essentially related to Zn hyperaccumulation (Weber et al., 2004), while it seems to be involved in Ni hyperaccumulation in *T. caerulescens* where a strong correlation occurs between NA and Ni but not between NA and Zn (Vacchina et al., 2003; Callahan et al., 2007). The transport of NA-metal complexes, required for loading into and unloading from vascular tissues, is mediated by the YSL (Yellow Strip 1-Like) family of proteins (Colangelo and Gueriot, 2006). Transcriptomic studies led three YSL transporter genes to be identified, namely *TcYSL3*, *TcYSL5*, and *TcYSL7*, constitutively overexpressed in roots and shoots of *T. caerulescens*, with the expression of *TcYSL3* and *TcYSL7* localized around the root vascular tissues. Evidence supports a role of these YSLs in metal hyperaccumulation by participating in vascular transport of NA-metal (particularly NA-Ni) complexes (Gendre et al., 2006). In roots of the Ni hyperaccumulator *Alyssum lesbiacum* a His-coupled



Ni xylem loading occurs and the Ni-His complexation is favored by a high endogenous His pool (Krämer et al., 1996; Kerkeb and Krämer, 2003) due to the constitutively overexpression of ATP-phosphoribosyltransferase, the first enzyme of the His biosynthetic pathway (Ingle et al., 2005). Elevated free His concentrations have also been reported in roots of *T. geosingense* (Persans et al., 1999) and a Ni induced His accumulation has been observed in *T. caerulescens*, suggesting that a similar mechanism might operate in all these Ni-hyperaccumulating species (Assunção et al., 2003b). However, the transport system involved in Ni-His xylem loading has not been identified yet. Recent evidence suggests that the Ni-His complexation, besides sustaining the heavy metal release into the xylem, may also prevent the trapping of Ni in the root cell vacuoles maintaining the ions in a detoxified form available for translocation to the shoots (Verbruggen et al., 2009).

In arsenic hyperaccumulators, such as *P. vittata* and *Pteris cretica*, the higher rate of uptake into the roots and the lower sequestration within the cell vacuoles contribute to the greater As translocation to the shoot, when compared with non accumulating ferns (Poynton et al., 2004). A percentage of As taken up as arsenate ( $\text{As}^{\text{V}}$ ) is rapidly reduced to arsenite in the root cells and translocated to the shoot as  $\text{As}^{\text{III}}$  (Poynton et al., 2004; Vetterlein et al., 2009). In the xylem sap of *P. vittata*  $\text{As}^{\text{III}}$  accounts for 93%–98% of the total As (Su et al., 2008), in agreement with the finding by Duan et al. (2005) of arsenate reductase activity only in the roots of the hyperaccumulating fern. The reduction of  $\text{As}^{\text{V}}$  to  $\text{As}^{\text{III}}$  renders necessary transport systems other than that for phosphate to translocate arsenite to the shoot, and the most likely candidates seem to be aquaporins that are known to mediate transmembrane  $\text{As}^{\text{III}}$  transport in mammals (Liu et al., 2002) and yeasts (Wysocki et al., 2001). Indeed, plasma membrane-localized aquaglyceroporins of NIP (Nodulin 26-like Intrinsic Protein) subfamily, permeable to arsenite but not to arsenate, have recently been shown to operate in the  $\text{As}^{\text{III}}$  transport into the xylem in plants (Ma et al., 2008; Kamiya et al., 2009). It is conceivable that a high expression of this kind of transporters might account for the arsenite efflux from root cortical cells toward xylem in As hyperaccumulators (Zhao et al., 2009). Differently, in Se hyperaccumulator plants such as *A. bisulcatus* and *Stanleya pinnata* most of Se uptaken into the roots is translocated to the shoots as selenate through the sulfate transport systems (Sors et al., 2005a), the young leaves being the major site for  $\text{SeO}_4^{2-}$  reduction and metabolism (Freeman et al., 2006).

#### 25.6.4 HEAVY METAL DETOXIFICATION IN LEAVES

The concentration of huge quantities of heavy metals (or metalloids) in the aboveground parts (especially leaves) in hyperaccumulating plants without suffering from phytotoxicity requires the efficient activity of detoxification mechanisms in the cells mainly based on metal complexation with ligands and/or sequestration in extracytosolic sites, such as vacuoles or cell walls (Küpper et al., 2001; Cosio et al., 2005). The ability of hyperaccumulators to remove high heavy metal amounts from active cellular sites by storing them in inactive compartments is a key mechanism of tolerance which relies, at least in part, on the constitutive overexpression of transporters localized at the cell tonoplast and/or plasma membrane.

A role in heavy metal hyperaccumulation has been recognized for CDFs (Cation Diffusion Facilitators), also called MTPs (Metal Transporter Proteins), a class of proteins which mediate bivalent cation efflux from the cytosol (Mäser et al., 2001). It has been found that the transcript levels of the MTP gene *ZTP1* are higher in leaves of *T. caerulescens* compared with *T. arvense* (Assunção et al., 2001; Hammond et al., 2006), and that the tonoplast MTP1 protein, with a plausible role in vacuolar Zn sequestration, is more expressed in shoots of *A. halleri* than in the Zn-sensitive *Arabidopsis lyrata* (Dräger et al., 2004). Furthermore, in the Zn/Ni hyperaccumulator *T. geosingense* the localization of the constitutively overexpressed TgMTP1 at the tonoplast and plasma membrane is consistent with its role in both Zn and Ni import into the shoot vacuoles (Persans et al., 2001) and export from the cytoplasm to the cell wall (Kim et al., 2004). Comparative transcriptome analyses between shoots of *A. halleri* and *A. thaliana* (Becher et al., 2004) and *T. caerulescens* and *T. arvense* (Hammond et al., 2006) suggest the involvement in Zn hyperaccumulation of HMA3,

a vacuolar P<sub>IB</sub>-type-ATPase overexpressed in the hyperaccumulating species. In leaves of *A. halleri* Elbaz et al. (2006) found a constitutively high expression of MHX, a vacuolar metal/proton exchanger belonging to the superfamily of Ca<sup>2+</sup>/cation (CaCA) exporters. Moreover, constitutively overexpressed genes coding for some cation exchangers (CAXs) of another CaCA subfamily have been identified in *A. halleri* and *T. caerulescens* and are supposed to mediate the Cd compartmentation (Cracium et al., 2006; van de Mortel, 2006, 2008). More recently the activity of a vacuolar Ni/H<sup>+</sup> antiport has also been evidenced by Ingle et al. (2008) in leaves of the Ni hyperaccumulator *Alyssum lesbiacum*. All these classes of transporters are important for heavy metal sequestration and detoxification, thereby generating a metal sink in the shoot that could be one of the driving forces in metal hyperaccumulation (Becher et al., 2004).

A further strategy to detoxify heavy metals in leaves of hyperaccumulating plants is by complexing them in the cytoplasm with low molecular weight chelators, principally organic acids, to avoid binding to physiologically active proteins and to facilitate the subsequent transport and storage within vacuoles. Cd is mainly sequestered as malate complexes in leaves of *T. caerulescens* (Ueno et al., 2008) while the majority of Zn is bound to citrate in this plant (Salt et al., 1999) and to malate in *A. halleri* (Sarret et al., 2002). Citrate is the main Ni ligand in a range of *Alyssum* hyperaccumulating species (Lee et al., 1978) and in *T. goesingense* (Krämer et al., 2000). Hitherto the identity of transporters mediating the vacuolar storage of the chelated heavy metals remains unknown.

Differently from nonhyperaccumulating plants the detoxification strategies in hyperaccumulators do not rely on heavy metal binding with high molecular mass chelators, such as phytochelatins (Ebbs et al., 2002; Schat et al., 2002). As regards metallothioneins (MT), an overexpression of some members of the MT gene family has been noticed in high Cd hyperaccumulating populations of *T. caerulescens* (Roosens et al., 2005; Hassinen et al., 2007). However, no clues exist currently about a role in heavy metal hypertolerance of these MTs, which appear to be more involved in Cu homeostasis in the context of exceptional Cd and Zn concentrations in the hyperaccumulator tissues (Roosens et al., 2005).

In various *Thlaspi* species, the ability to hyperaccumulate Ni is related to high glutathione levels in shoot tissues, driven by constitutive activation of enzymes of sulfur assimilation pathway and GSH metabolism (Freeman et al., 2004). This enhanced GSH synthesis, also induced in shoots of *T. caerulescens* (van de Mortel et al., 2008) and *S. alfredii* (Sun et al., 2007) by Cd exposure, suggests an involvement of GSH antioxidant activity in hyperaccumulation. Higher antioxidation-related gene expressions (Chiang et al., 2006) and antioxidative enzyme activities (Table 25.1) (Schickler and Caspi, 1999; Wang et al., 2008) occur in other Ni and Cd hyperaccumulators compared with relative nonhyperaccumulating species.

The essential role played in hyperaccumulation strategies by the heavy metal exclusion from metabolically active sites of leaves accounts for their preferential sequestration in leaf epidermis where they do least damage to photosynthesis. In leaves of *T. caerulescens*, the highest Zn and Cd concentrations are present in the vacuolar sap of the epidermal cells (Küpper et al., 1999; Ma et al., 2005) but, interestingly, Zn appears to be largely absent from within the subsidiary and guard cells of the stomatal complexes, plausibly to protect the function of stomata against metal toxicity (Frey et al., 2000; Cosio et al., 2005). A similar absence of Ni in the guard cells has been reported in the Ni hyperaccumulator *Thlaspi montanus* by Healt et al. (1997) who suggest that the heavy metal exclusion might be due to unique ion-transport properties of the guard cell plasma membrane. A lower Ni concentration in the stomatal complex than in the other epidermal cells also occurs in the hyperaccumulators *T. goesingense* (Küpper et al., 2001) and *Alyssum murale* (Broadhurst et al., 2004). Different ecotypes of *T. caerulescens* accumulate Cd both inside the vacuoles and in the cell walls of the epidermis (Cosio et al., 2005) while in *A. halleri* the highest concentrations of Cd and Zn are found in the leaf trichomes (Küpper et al., 2000). Ni was distributed predominantly in vacuoles of the leaf epidermal cells also in several hyperaccumulator species of *Alyssum* (Küpper et al., 2001; Broadhurst et al. 2004; Asemaneh et al., 2006) and in *Hybanthus floribundus* (Bidwell et al., 2004). The Ni hyperaccumulator *Berkheya coddii* behaves in a peculiar manner in that it accumulates Ni

at significantly higher concentrations in the cuticle of the upper epidermis than in the rest of the leaf thus protecting the mesophyll cytoplasm from the heavy metal toxic effects (Robinson et al., 2003).

In fronds of the arsenic hyperaccumulator fern *P. vittata* up to 90% of the total As is present as inorganic arsenite stored in vacuoles (Pickering et al., 2006; Su et al., 2008). The vacuolar sequestration results as the key mechanism of As detoxification in the hypertolerant ferns, but how it occurs and what the As transporters are at the tonoplast deserve further investigation (Zhao et al., 2009). Also in As hyperaccumulators, differently from nonhyperaccumulators, phytochelatins do not seem to have a significant role in As detoxification (Zhang et al., 2004; Vetterlein et al., 2009). Raab et al. (2004) showed that less than 1% of the As is complexed with phytochelatins in fronds of *P. cretica*. According to Zhao et al. (2002a), this might be explained with the prohibitive cost of a PC-dependent As hyperaccumulation, which would require a S amount exceeding that normally accumulated inside the plants. In the As tolerance of hyperaccumulating ferns, instead, an inherently high antioxidant potential plays a role against ROS produced by arsenate to arsenite reduction. This is based on an ascorbate-GSH pool (Sing et al., 2006) and an antioxidative enzyme activity (Table 25.1) much greater than in nonhyperaccumulating species (Srivastava et al., 2005; Kertulis-Tartar et al., 2009).

As regards the selenate, it is known that its assimilation, which occurs in plastids through the sulfur assimilation pathway, leads to formation of selenoamino acids, such as selenocysteine (Se-Cys), whose misincorporation into proteins accounts for the Se toxicity (Terry et al., 2000). Thus, in hyperaccumulator plants the ability to tolerate very high Se concentrations in leaves relies on the activity of the enzyme selenocysteine methyltransferase (SMT) that specifically methylates SeCys to produce the harmless nonprotein amino acid methylselenocysteine (MeSeCys). The SMT is constitutively expressed in roots and leaves of Se hyperaccumulators (Pickering et al., 2003) and the SMT activity is closely correlated with the Se hyperaccumulation in species with varying capacity to accumulate Se (Sors et al., 2005a). In the young leaves of the hyperaccumulator *A. bisulcatus* containing the greatest amount of Se, the MeSeCys accounts for 99% of the accumulated Se (Pickering et al., 2003). Conversely, in the non-Se-hyperaccumulators, such as *Astragalus drummondii*, the synthesis of MeSeCys cannot occur due to the inability of a SMT-like enzyme to catalyze the SeCys methylation (Sors et al., 2009). Interestingly, also Se, like the heavy metals, is concentrated predominantly in the leaf epidermis of hyperaccumulators, while in nonhyperaccumulators it is distributed throughout the mesophyll cells (Freeman et al., 2006). This finding strongly suggests that actively directed transport mechanisms for hyperaccumulated elements may be a unifying theme in hyperaccumulating species (Freeman et al., 2006).

## 25.7 CONCLUSIONS

Chemical, technological, and agricultural activities pour into the environment ever greater quantities of heavy metals thus polluting wild and cultivated lands and exposing plants growing on poisoned soils to the risk of phytotoxic damage. Moreover, the possible entrance of these heavy metals into the food chain via plants represents a serious threat to animals and human health.

The serious problem of heavy metal pollution led, in the last two decades, to a real explosion of research dealing with the harmful effects of heavy metals in plants and with the defense strategies that plants accomplish to tolerate this kind of dangerous chemicals. Great interest was also taken in highlighting the mechanisms allowing metalcolous species to hypertolerate and hyperaccumulate heavy metals. This because of the potential application of the acquired knowledge in strategies useful for removing unwanted heavy metals from the biosphere as well as for obtaining biofortified food crops.

The information gained led to a substantial clarification of physiological, biochemical, molecular, and genetic mechanisms of heavy metal tolerance in both excluder and hyperaccumulator plants, but, despite the recent progress achieved by the application of new technological methods, a number of “tesserae” are still waiting to be unraveled in order to complete the whole puzzle of metal tolerance. However, the great and increasing scientific and applied interest will surely allow most black boxes of this fascinating research field to be unlocked in the next few years.

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# 26 Heavy Metals and Plastid Metabolism

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## 26.1 INTRODUCTION

Heavy metals (HMs) are in general defined as metals with a specific gravity greater than 5.0 or with high atomic mass. Often, toxicity is associated with their definition; however, several HMs are necessary for the proper functioning and metabolism of living organisms. Such elements essential for plant development are Cu, Co, Fe, Mn, Mo, Ni, and Zn. One type of plant stresses is nutrient deficiency, with which most plants can barely cope; therefore their growth and crop productivity are impaired when the phytoavailability of any of these metals is low. Unfortunately, in several agricultural fields, essential-metal deficiency is a serious concern, especially in the case of Fe and Zn (Guerinot 2000). Another plant stress is represented by the excess of essential HMs and by the presence of nonessential metals. These nonessential HMs (e.g., Cd, Cr, Hg, Pb) are in general toxic or neutral for plant metabolism. The increased presence of HMs in air, soil, and water is also a global problem that represents a growing threat to the environment and to humankind and requires immediate attention. There are hundreds of natural and anthropologic sources of HMs, including industry, atmospheric deposition, use of agrochemicals, and waste disposal. The pollutants can enter plants via various mechanisms and in this way can easily reach intracellular compartments of plants, such as plastids. In this chapter, the stress caused by essential-HM deficiency and by excess

of HMs on plastid metabolism is discussed. In addition, the interesting phenomena of HM stress alleviation by the excess of another HM and the stimulatory effect of low concentration stressors on photosynthesis is briefly reviewed.

## 26.2 METALS INSIDE PLANTS

For healthy plant growth and development, a range of HMs must be acquired from the soil, transported along the plant, distributed, and compartmentalized in different tissues and cells. Clearly, membrane transport systems are likely to play a key role in these events. The genes encoding these plant nutrient transporters appear to be transcriptionally regulated by a feedback mechanism that reduces their expression when the plant reaches an optimal level of nutrition (reviewed by Amtmann and Blatt 2009, Burkhead et al. 2009, Kawachi et al. 2009). In most terrestrial plants, metals are absorbed by the roots and transported via the xylem into the aerial parts. Essential HMs are in general highly mobile and therefore approximately one third of them is translocated to the aboveground organs, while two-thirds are retained in the roots (e.g., Kawachi et al. 2009). However, when present or added in excess, most of the surplus essential metal(s) also accumulates in the roots, similar to most nonessential or toxic metals (e.g., Brunner et al. 2008, Zehra et al. 2009), but rarely the contaminating metal principally accumulates in the leaves (e.g., Vázquez et al. 1990). This way, in most cases, the root plastids are the first targets of HM toxicity or deficiency. Therefore, root plastids are in general less prone to metal deficiency, but more often affected by high levels of metals.

Aquatic plants and some epiphytes absorb the essential nutrients via their whole surface from the water or the air, respectively. In these cases, HMs may reach the plastids directly. Similarly, atmospheric deposition of pollutants contributes to toxic metal levels in aerial plant parts (leaves, fruits, flower parts, and stems) of terrestrial plants, and in this case, chloroplasts or other types of plastids (i.e., chromoplasts, amyloplasts) can be directly affected by relatively high concentrations of HMs, although not much is known about the uptake mechanisms and transportation of airborne metal pollutants within plant tissues.

Nonessential metals (Cd, Ag, and Pb), generally intrude into plant cells or into organelles at the expense of essential inorganic ions on account of similar properties, such as ionic radii (Perfus-Barbeoch et al. 2002), for example, Cd uses Ca ion channels to enter plant cells.

### 26.2.1 METAL TRANSPORTERS

After the first phase of extracellular metal adsorption—rapid and nonspecific binding of the cations to the negatively charged cell wall components and mobilization of the soil-bound HMs by secretion of organic acids by the roots—metals have to enter the symplasm by metal transporters or ion channels (Table 26.1). Most of the metals enter cells as cationic elements (e.g.,  $\text{Zn}^{2+}$ ), whereas others cross the plasma membrane as anionic groups (e.g.,  $\text{AsO}_4^{3-}$ ) or included in small organic compounds (e.g., methyl-mercury,  $\text{CH}_3\text{Hg}^+$ ) (reviewed by Kucera et al. 2008). The HMs are taken up as hydrated ions or in metal–chelate complexes through channel proteins and/or carrier proteins. These include the (1) heavy-metal ( $\text{P}_{1b}$ -type or CPx-type) ATPases (HMA) that can pump a variety of essential and nonessential HMs across the plasma membrane; (2) the natural resistance associated macrophage proteins (NRAMP), which are  $\text{H}^+$ -coupled transporters implicated in the transport of divalent ions (e.g.,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cd}^{2+}$ ); (3) the cation diffusion facilitators (CDFs) involved in  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Cd}^{2+}$  transport (Williams et al. 2000); (4) the ZRT, IRT-like protein (ZIP) family transporting mostly  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Zn}^{2+}$  (Cohen et al. 1998, Guerinot 2000, reviewed by Grotz and Guerinot 2006); (5) the cation/ $\text{H}^+$  antiporters exchanging  $\text{Na}^+$  or  $\text{H}^+$  to  $\text{Ca}^{2+}$  or  $\text{Cd}^{2+}$ ; and (6) ATP-binding cassette (ABC) transporters that have a role in intracellular transport of Cd, Fe, or Mn (Table 26.1, reviewed by Clemens 2001, Hall and Williams 2003, Kucera et al. 2008, Poirier et al. 2008). Although these transporters are rather specific for a single element, their specificity is not absolute, and other metals can also be transported (Table 26.1). However, knowledge about

**TABLE 26.1**

**Heavy-Metal Transporters and Ion Channels Characterized In Vivo or In Vitro and Localized Experimentally or Only Computer Predicted to Different Plant Membranes**

| Carrier Type                                      | Carrier            | Proposed Metal Specificity<br>(++ Indicates the Primarily Transported Metal) |    |    |    |    |    |    |    | Localization | References                                                                                                |
|---------------------------------------------------|--------------------|------------------------------------------------------------------------------|----|----|----|----|----|----|----|--------------|-----------------------------------------------------------------------------------------------------------|
|                                                   |                    | Cd                                                                           | Co | Cu | Fe | Mn | Ni | Pb | Zn |              |                                                                                                           |
| ABC-type transporter                              | STA1               |                                                                              |    |    | +  |    |    |    |    | M            | Briat et al. (2007)                                                                                       |
| Cation diffusion<br>facilitator (CDF)             | ZAT1/MTP1 and MTP3 |                                                                              |    |    |    |    |    |    | +  | T            | Van der Zaal et al. (1999), Broadley et al. (2007), Puig and Penarrubia (2009), and Kawachi et al. (2009) |
| Cation/H <sup>+</sup> antiporters                 | ShMTP1             |                                                                              |    |    |    | +  |    |    |    | T            | Delhaize et al. (2003)                                                                                    |
|                                                   | CAX2 (Ca)          | +                                                                            |    |    |    | +  |    |    |    | T            | Hirschi et al. (2000)                                                                                     |
|                                                   | CAX4 (Ca)          |                                                                              |    |    |    | +  | +  |    |    | T            | Cheng et al. (2002)                                                                                       |
| Conserved copper<br>transporter (COPT)            | COPT1, COPT2       |                                                                              |    | +  |    |    |    |    |    | PM           | Sancenon et al. (2003), Puig et al. (2007), and Burkhead et al. (2009)                                    |
|                                                   | COPT?              |                                                                              |    | +  |    |    |    |    |    | T            | Yruela (2009)                                                                                             |
|                                                   | COPT?              |                                                                              |    | ++ |    |    |    |    | +  | PE           | Sancenon et al. (2003), Burkhead et al. (2009), and Yruela (2009)                                         |
| Cyclic nucleotide gated<br>channels (shaker type) | CNGC1              |                                                                              |    |    |    |    | ?  | +  |    | PM           | Sunkar et al. (2000)                                                                                      |
|                                                   | NtCBP4             |                                                                              |    |    |    |    | ++ | +  |    | PM           | Arazi et al. (1999) and Talke et al. (2003)                                                               |
| Heavy-metal ATPases<br>(HMA)                      | HMA1               | +                                                                            | +  | ++ |    |    |    |    | ++ | PE           | Seigneurin-Berny et al. (2006), Yruela (2009), and Kim et al. (2009)                                      |
|                                                   | HMA2               | +                                                                            | +  | +  |    |    | +  | +  | ++ | PM           | Eren and Argüello (2004)                                                                                  |
|                                                   | HMA3               | +                                                                            | +  |    |    |    |    | +  | ++ | T            | Pilon et al. (2009) and Morel et al. (2009)                                                               |
|                                                   | HMA4               | +                                                                            |    |    |    |    |    |    | ++ | PM           | Eren and Argüello (2004)                                                                                  |
|                                                   | HMA5               |                                                                              |    | +  |    |    |    |    |    | PM, G        | Andres-Colas et al. (2006) and Yruela (2009)                                                              |

(continued)

**TABLE 26.1 (continued)**  
**Heavy-Metal Transporters and Ion Channels Characterized In Vivo or In Vitro and Localized Experimentally or Only Computer Predicted to Different Plant Membranes**

| Carrier Type                                                   | Carrier         | Proposed Metal Specificity<br>(++ Indicates the Primarily Transported Metal) |    |    |    |    |    |    |    | Localization | References                                                                                               |
|----------------------------------------------------------------|-----------------|------------------------------------------------------------------------------|----|----|----|----|----|----|----|--------------|----------------------------------------------------------------------------------------------------------|
|                                                                |                 | Cd                                                                           | Co | Cu | Fe | Mn | Ni | Pb | Zn |              |                                                                                                          |
| Low-affinity cation transporter                                | HMA6/PAA1       |                                                                              |    | ++ |    |    |    |    | +  | PE           | Shikanai et al. (2003), Abdel-Ghany et al. (2005), Puig et al. (2007), and Yruela (2009)                 |
|                                                                | HMA8/PAA2       |                                                                              |    | +  |    |    |    |    |    | PI           | Shikanai et al. (2003), Puig et al. (2007), and Burkhead et al. (2009)                                   |
|                                                                | OsHMA9          |                                                                              |    | +  |    |    |    | +  | +  | PM           | Lee et al. (2007)                                                                                        |
|                                                                | RAN1/ HMA7      |                                                                              |    | +  |    |    |    |    |    | ER, PG       | Yruela (2009) and Kim et al. (2009)                                                                      |
|                                                                | TaLCT1 (Ca)     | +                                                                            |    |    |    |    |    |    |    | PM           | Schachtman et al. (1997), Antosiewicz and Hennig (2004), Wojas et al. (2007), and Szczerba et al. (2009) |
| Magnesium-selective ion channel                                | MGT1 (Mg)       |                                                                              | +  | +  | +  | +  | +  |    |    | PM           | Li et al. (2001)                                                                                         |
| Mg <sup>2+</sup> (Zn <sup>2+</sup> )/H <sup>+</sup> antiporter | MHX (Mg)        | +                                                                            |    |    | +  |    |    |    | +  | T            | Shaul et al. (1999) and Berezin et al. (2008)                                                            |
| NRAMP                                                          | NRAMP1          | +                                                                            |    |    | ++ | +  |    |    |    | PE           | Curie et al. (2000), Thomine et al. (2000), Grotz and Guerinot (2006), and Puig et al. (2007)            |
|                                                                | NRAMP3 TcNRAMP3 | +                                                                            |    |    | ++ |    |    |    | +  | T            | Grotz and Guerinot (2006) and Oomen et al. (2009)                                                        |

|                                |                 |   |   |       |       |   |       |     |                                                                                                        |
|--------------------------------|-----------------|---|---|-------|-------|---|-------|-----|--------------------------------------------------------------------------------------------------------|
|                                | NRAMP4 TcNRAMP4 | + |   | +     | +     |   | +     | T   | Hall and Williams (2003), Grotz and Guerinot, (2006), and Oomen et al. (2009)                          |
| Oligopeptide transporter (OPT) | YSL1, YSL3      |   |   | Cu-NA | Fe-NA |   | Zn-NA | PM  | Waters and Grusak (2008)                                                                               |
| Permease                       | ZmYS1           |   |   |       | Fe-PS |   |       | PM  | Curie et al. (2001)                                                                                    |
|                                | PIC1            |   |   |       | +     |   |       | PE  | Duy et al. (2007) and Puig and Penarrubia (2009)                                                       |
| Vacuolar iron transporter      | VIT1            |   |   |       | +     |   |       | T   | Kim et al. (2006) and Briat et al. (2007)                                                              |
| ZRT, IRT-like proteins (ZIP)   | IRT1            | + | + |       | ++    | + | +     | PM  | Cohen et al. (1998), Connolly et al. (2002), Fodor (2006), Puig et al. (2007), and Mills et al. (2008) |
|                                | IRT3            |   |   |       |       | + | +     | PM  | Broadley et al. (2007)                                                                                 |
|                                | ZIP2 and ZIP4   |   |   | +     |       |   | ±     | PM? | Burkhead et al. (2009)                                                                                 |
|                                | ZIP4            |   |   | +     |       |   | +     | PE  | Grotz et al. (1998), Hall and Williams (2003), and Grotz and Guerinot (2008)                           |

*Notes:* Carriers are listed in alphabetic order. For more details see the cited references. ++ indicates the main transported HM, and when the transporter is primarily responsible for the transport of another (non-heavy) metal, the symbol of the metal is indicated in parenthesis after the name of the carrier. *Abbreviations:* ABC, ATP-binding cassette; ER, endoplasmic reticulum; G, Golgi; M, mitochondrial membrane; NA, nicotianamine; PE, plastid envelope; PG, post-Golgi membranes; PI, plastid inner membranes; PM, plasma membrane; PS, phytosiderophore; T, tonoplast.

? Question marks indicate uncertainties.

these transporters is still rather scarce and many of them are only characterized *in vitro*. Some of them are expressed in an organ-specific manner, and their intracellular localization also varies, i.e., some are located in the plasma membrane, others are involved in other endomembranes such as chloroplast or mitochondrial envelopes, Golgi, and tonoplast membranes (Table 26.1, reviewed by Hall and Williams 2003). As a consequence, some of them have a role in metal uptake, extracellular detoxification, or long-distance transportation of HMs within the plants (from those located in the plasma membrane); others have roles in the sequestration of excess HMs in the vacuole or in their remobilization from this organelle (these are found in the tonoplast) or in metal delivery to other intracellular compartments.

While transporters require active transport through symporters and antiporters, and can transport ions against an electrochemical potential gradient, passive ion transport occurs through channels, which are membrane proteins with ion-selective pores that allow ion movement down an electrochemical gradient. Among cationic channels, some are not highly selective and can therefore participate in the transport of different toxic cations (reviewed by Demidchik et al. 2002, Table 26.1).

### 26.2.2 INTERACTIONS BETWEEN DIFFERENT METALS

The different HMs can interact in the soil. Positive and negative synergisms, competition, protection, and sequential additivity are observed among the interactions. The nature of interactions varies considerably with concentration levels, soil pH, soil texture, level of soluble Ca in soil, presence of salinity, differential distribution in soil of the metals present in high quantities, presence or absence of chelating agents, soil organic matter levels, and other factors (reviewed by Wallace et al. 1992). HMs bind organic ligands with different stability constants or may form precipitates with inorganic anions in the soil solution of the rhizosphere. This way, they might mutually influence each other's solubility or compete for different binding ligands, including those secreted out by the plant to improve the solubility of essential metals (e.g., phytosiderophores for Fe; Delhaize and Ryan 1995, Hinsinger et al. 2003). As illustrated in Table 26.1, some HMs use the same ion channels, metal transporters or chelators, and therefore they have an impact on each other's uptake and intracellular concentration. Therefore, toxic HMs often cause reduced productivity and biomass in crop plants indirectly, by inducing essential-metal deficiency in the plants.

It is generally assumed that for nonessential elements such as Cd, there are no specific uptake mechanisms. Cd ions compete with nutrients such as K, Ca, Mg, Fe, Mn, Cu, Zn, and Ni (Table 26.1, reviewed by Pál et al. 2006, Clemens et al. 2009). Some data suggest that Cd can be taken up via the phytosiderophore pathway as well (reviewed by Reichman and Parker 2005, Fodor 2006). This outlines that, besides alleviating nutrient deficiency of Fe, Zn or Ni, phytosiderophores also increase the bioavailability of toxic metals and thus increase the potential for food-chain transfer hazards for them (e.g., for Cd), and they also increase the competition between Fe and other metals, leading then to physiological Fe-deficiency. In Cd-treated leaves, Cd can enter guard cells via Ca channels (Perfus-Barbeoch et al. 2002). It was also observed that Pb can enter the cells via Ca and Ni transport systems (Table 26.1, Arazi et al. 1999, Sunkar et al. 2000, Wojas et al. 2007). However, data are relatively scarce in the literature about the uptake of nonessential HMs.

Besides the competition of metals in the soil and during metal uptake in the roots, HMs also compete in their translocation from the roots to the shoots. Since very little metal in plants is assumed to exist as free ions, several small organic molecules have to be implicated in metal ion homeostasis as metal ion ligands or chelators, in order to improve acquisition and transport of metal ions with low solubility and to enhance immobilization for metal tolerance and storage. Citrate, mugineic acid, avenic acid, deoxymugineic acid (Suzuki et al. 2008) and nicotianamine have been shown to participate in the intra- and intercellular transport of essential metals such as Cu, Fe, Mn, Ni, or Zn (reviewed by Fodor 2006, Puig et al. 2007, Chen et al. 2009, Yruela 2009). *In vitro*, nicotianamine is able to form stable complexes with Mn, Fe, Co, Zn, Ni, and Cu, in increasing order of affinity (Curie et al. 2009). The pH stability of these complexes suggests their occurrence in symplasm or



apoplasm, indicating that nicotianamine should complex Cu, Fe, and Zn in the phloem, and Cu and Zn in the xylem for their translocation from roots to shoots (reviewed by Yruela 2009).

The Zn/Cd-transporting ATPases, HMA2 and HMA4, essential for root-to-shoot Zn translocation, facilitate the transport of Cd (Table 26.1, Wong and Cobbett 2009). Citrate was found to be the principal compound chelating metals in the xylem sap (Cd: reviewed by Fodor 2006, Hasan et al. 2009; Pb: reviewed by Fodor 2002, 2006).

Finally, after their xylem unloading, essential HMs must enter the symplasm of the cells in the aerial parts of the plant by different membrane transport systems (Table 26.1) and have to reach the intracellular compartment where they will be used. The mechanisms of intracellular HM trafficking is still not very well understood. In the next part of the chapter, we review the effect of unbalanced metal concentrations on the structure and function of different plastids.

### 26.3 METALS AND PLASTID METABOLISM

The plastid is a unique, semiautonomic organelle characteristic of photosynthetic eukaryotic cells and evolved from the endosymbiosis of free-living cyanobacteria with an ancient eukaryotic cell (reviewed by Solymosi and Schoefs 2008). All plants contain plastids. These organelles are widespread within the plants, because with a few exceptions, all cells possess plastids in one form or another. Despite their diversity, plastids have several common features. Their boundary to the cytoplasm is a double membrane called the plastid envelope, which encircles the protein-rich stroma. The outer membrane is permeable to molecules up to a molecular mass of ca. 6 kDa due to the presence of porins, while the inner membrane is highly selective and contains different membrane transporters (e.g., Table 26.1, reviewed by Weber et al. 2005, Johnson et al. 2006, Aronsson and Jarvis 2008). Besides the more or less developed inner membrane system, the plastids often contain spherical bodies that contain lipids, carotenoids, plastoquinone, and proteins and others that are called plastoglobuli that contain lipids, carotenoids, plastoquinone, and proteins (Austin et al. 2006). In addition, the plastids contain nucleoids (DNA-containing structures), procaryotic ribosomes and, as semiautonomic organelles, they can synthesize at least part of their proteins. Different inclusions are also frequently seen in the stroma of the plastids. One example for them is the Fe-containing phytoferritin or simply ferritin (reviewed by Briat et al. 1999).

Depending on their physiological function, chemical composition and internal structure, the plastids are divided into different groups (reviewed by Solymosi and Schoefs 2008). Chloroplasts are present in many different types of cells and organs (i.e., in ripening or mature fruits, in green colored parts of flowers like the calyx and the gynoecium, in green stems and leaves). Their presence is essential because they provide energy and oxygen to the biosphere via photosynthesis. Their inner membrane system is laterally segregated into two major functional domains, the appressed (stacked) granal membranes and the interconnected, non-appressed stromal membranes (reviewed by Solymosi and Schoefs 2008).

Besides chloroplasts, several nongreen plastid types exist and are developmentally interrelated. Proplastids are characteristic in meristematic cells (e.g., in the root and shoot apical meristems), and in dedifferentiated and/or reproductive cells (reviewed by Solymosi and Schoefs 2008). Proplastids are small and have only a poorly developed inner membrane structure. They can differentiate into any other plastid type. Under natural light conditions, and in photosynthesizing organs, they differentiate into chloroplasts. However, in angiosperm plants, in the absence of light, normal chloroplast development is impaired, and proplastids differentiate to so-called etioplasts (e.g., Solymosi et al. 2004, 2006a, 2007, reviewed by Solymosi and Schoefs 2008). The formation of etioplasts is a natural phenomenon observed in different crops (e.g., cabbage heads—Solymosi et al. 2004) and in seeds germinating in the soil in agricultural systems (e.g., sunflower: Solymosi et al. 2007; bean: Schoefs and Franck 2008, reviewed by Solymosi and Schoefs 2008). These plastids contain special inner membrane system consisting of lamellar prothylakoids and a paracrystalline membrane network called prolamellar body.

During senescence of the tissues, and also before defoliation of the leaves, the conversion of chloroplasts to the so-called gerontoplasts (or senescing chloroplasts) can be observed (reviewed by Thomas 1997). During this transformation, chlorophyll (Chl) is degraded, the inner membrane system of the chloroplasts is disorganized and large, often electron-transparent plastoglobuli appear (e.g., Solymosi et al. 2004).

The leucoplasts are colorless plastids with poorly developed inner membranes; they are specialized in storage of either starch (amyloplasts) or lipids (elaioplasts) or proteins (proteinoplasts) and function therefore as storage organelles. These plastids are heterotrophic and convert photosynthates derived from source tissues into storage compounds. Amyloplasts are characteristic of the parenchymatic tissues of storage organs (tubers, rhizomes, roots, fruits, seeds) but can also be found in root cap cells, where they are associated with geotropism (reviewed Solymosi and Schoefs 2008).

The chromoplasts accumulate carotenoids and are responsible for the bright yellow, orange, and red colors of petals, fruits like tomatoes, pepper, roseberry and for that of some roots, i.e., carrot (reviewed by Solymosi and Schoefs 2008).

Thus, in addition to photosynthesis, plastids harbor many more vital biosynthetic functions, such as nitrogen and sulfur assimilation or the biosynthesis of fatty acids, (aromatic) amino acids, lipids, pigments (Chls and carotenoids), purines, pyrimidines and secondary metabolites including terpenoids, and other important compounds used in pharmaceutical or perfume industry. In consequence, these functions require an active solute exchange across the outer and inner envelope membranes surrounding the chloroplast stroma (reviewed by Weber et al. 2005). Metal transport proteins in both membrane systems thus provide a bottleneck to the control of metal homeostasis in the chloroplast as well as in the plant cell (Table 26.1).

The transition metals Fe, Cu, and Mn play a vital role in photosynthetic electron transport in chloroplasts and in stroma-located reactions of CO<sub>2</sub> fixation (Table 26.2). Plastid localized Fe and Cu/Zn superoxide dismutases scavenge reactive oxygen species (ROS). In addition, Zn is known to function as a cofactor (in RNA polymerase, Zn finger domains) in plastid transcription (Table 26.2), while among others, Fe is required for heme and for Fe-S clusters (Cornah et al. 2002) and for enzymes of Chl biosynthesis (Myśliwa-Kurdziel and Strzałka 2002, Duy et al. 2007). Several other enzymes functioning in other plastid types also require HMs (Table 26.2).

Among all plastid types, the effect of metal stress and also the functioning of metal uptake machineries are best characterized in chloroplasts, but there are also a few data indicating changes induced by excess metals in other plastid types (for details see below). Both essential-metal deficiency and excess influence plastid metabolism. Similarly, various concentrations of nonessential metals also affect plastid structure and function. In this chapter, these processes are briefly summarized. Since the Chl concentration may fundamentally influence the functioning of the photosynthetic apparatus and thus affect the whole plant metabolism, it is a really important factor in assessing the impact of metal stress in chloroplasts and on plant productivity. One of the most usual symptoms of metal deficiency or metal excess is chlorosis, i.e., the decrease of the Chl content. This further outlines, that plastids—and especially chloroplasts—are central targets in metal stress.

### 26.3.1 IMPACT OF UNBALANCED METALS ON NONGREEN PLASTIDS

As discussed earlier, except in the case of airborne HMs in terrestrial or epiphytic plants, or water polluting elements for aquatic plants, HMs generally first affect the cells of roots or other underground organs (such as potato tubers, onion bulbs) and may interact with their plastids. Unfortunately, there are almost no data about the effect of HM deficiency or excess on nongreen plastids.

Although excess HMs and essential-metal deficiency also interfere with the metabolism of nongreen plastids, much less data are available on structural and metabolic alterations of these plastids (Table 26.3) than chloroplasts. For instance, besides chloroplasts, heme biosynthesis mostly occurs in root plastids and etioplasts, and is affected by Fe-deficiency (Cornah et al. 2002). Nongreen plastids play important roles in several important plant metabolic processes including amino acid

**TABLE 26.2**  
**Nonexhaustive List of Key Molecules Requiring Essential Metals**  
**in the Chloroplast**

| Metals | Proteins                                    | References               |
|--------|---------------------------------------------|--------------------------|
| Cu     | Cu/Zn-superoxide dismutase                  | Grace (1990)             |
|        | Cytochrome oxidase                          | Hänsch and Mendel (2009) |
|        | Plastocyanin                                | Abdel-Ghany (2009)       |
|        | Polyphenol oxidase                          | Kieselbach et al. (1998) |
| Fe     | Ascorbate peroxidase                        | Raven et al. (1999)      |
|        | Cytochrome b6-f                             | Raven et al. (1999)      |
|        | Cytochrome c6                               | Raven et al. (1999)      |
|        | Ferredoxin                                  | Tognetti et al. (2007)   |
|        | Ferredoxin–thioredoxin reductase            | Duy et al. (2007)        |
|        | Ferritin                                    | Briat et al. (1999)      |
|        | Ferrochelatase                              | Cornah et al. (2002)     |
|        | Fe-SOD                                      | Allen (1995)             |
|        | Glutamine-2-oxo-glutarate amido transferase | Duy et al. (2007)        |
|        | NADPH-plastoquinone oxidoreductase          | Raven et al. (1999)      |
|        | Nitrite reductase                           | Briat and Vert (2004)    |
|        | Pheophorbide <i>a</i> oxygenase             | Duy et al. (2007)        |
|        | Sirohydrochlorin ferrochelatase             | Duy et al. (2007)        |
|        | Sulfite reductase                           | Duy et al. (2007)        |
| Mg     | Tic55                                       | Duy et al. (2007)        |
|        | Chlorophylls                                | Shaul (2002)             |
| Mn     | Glutathione synthetase                      | Shaul (2002)             |
|        | Isocitrate dehydrogenase                    | Elias and Givan (1977)   |
| Mn     | Malic enzyme                                | Takeuchi et al. (2000)   |
|        | Mn-SOD                                      | Grace (1990)             |
|        | Phenylalanin ammonia lyase                  | Nishizawa et al. (1979)  |
|        | Water oxydase                               | Grace (1990)             |
| Mo     | Aldehyde oxidase                            | Weigel et al. (1986)     |
|        | Sulfite oxidase                             | Eilers et al. (2001)     |
|        | Xanthine dehydrogenase                      | Borner et al. (1986)     |
| Zn     | Carbonic anhydrase                          | Randall and Bouma (1973) |
|        | Cu/Zn-SOD                                   | Grace (1990)             |
|        | Enzymes involved in RNA editing             | Hänsch and Mendel (2009) |
|        | Metalloendopeptidase                        | Moberg et al. (2003)     |
|        | Stromal processing peptidase                | Hänsch and Mendel (2009) |
|        | Zn finger                                   | Sasaki et al. (1989)     |
|        | Zn-metalloprotease FtsH                     | Bailey et al. (2001)     |
|        | Zn-protease degrading RUBISCO               | Bushnell et al. (1993)   |

biosynthesis, hormone synthesis, sugar homeostasis, storage of different metabolites, carotenoid synthesis, and secretion. The secondary metabolite production of nongreen plastids is important in medicinal plants and crops also for human health and nutrition. Often, edible parts of crops contain nongreen plastids (e.g., carrot roots contain chromoplasts; celery, potato tubers, onion bulbs, garlic, and radish contain amyloplasts and/or proplastids; the inner tissues of cabbage heads, avocado and different cucumber fruits contain etioplasts or etio-chloroplasts) (reviewed by Solymosi and Schoefs 2008). However, molecular interactions of these plastids and the effect of metals on plastid metabolism in these organs remain poorly understood. Root plastids seem to be a primary target

**TABLE 26.3**  
**Examples of Alterations in the Ultrastructure of Nongreen Plastids Caused by Excess of Heavy Metals**

| Metal            | Species                                   | Plastid Alterations                                                                                                                                                                                                               | References                             |
|------------------|-------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|
| Cd               | Bean ( <i>Phaseolus vulgaris</i> )        | Decreased starch content and reduced internal membrane system in root plastids                                                                                                                                                    | Barceló et al. (1988)                  |
|                  |                                           | Ferritin-like deposits in root plastids                                                                                                                                                                                           | Vázquez et al. (1992)                  |
|                  | Maize ( <i>Zea mays</i> )                 | No ultrastructural effect on etioplasts and on etioplast–chloroplast conversion                                                                                                                                                   | Ghoshroy and Nadakavukaren (1990)      |
|                  | Soybean ( <i>Glycine max</i> )            | Disruption of the prolamellar body structure in etioplasts, retarded etioplast–chloroplast conversion (delayed grana formation, swollen intrathylakoidal spaces in greening plastids)                                             | Ghoshroy and Nadakavukaren (1990)      |
| Cr (VI)          | Bean ( <i>Phaseolus vulgaris</i> )        | Amoeboid plastids in root tip cells, appearance of amyloplasts in upper parts of the roots                                                                                                                                        | Vázquez et al. (1987)                  |
| Cu               | Oregano ( <i>Origanum vulgare</i> )       | Increased starch content in etio-chloroplasts of stems<br>Amyloplast-leucoplast transition is induced in roots, decrease in starch content, dark globular inclusions and many small droplets of an electron translucent substance | Panou-Filotheou and Bosabalidis (2004) |
| Hg               | Wheat ( <i>Triticum aestivum</i> )        | Changes in the unit cell size of prolamellar bodies (PLBs) of etioplasts, formation of vesicles on the PLB surface                                                                                                                | Solymosi et al. (2006b)                |
| <sup>99</sup> Tc | Common bean ( <i>Phaseolus vulgaris</i> ) | Amoeboid plastids, different forms of plastid inclusions (“engulfment” of cytoplasm or a mitochondrion)                                                                                                                           | Vázquez et al. (1990)                  |

of metal excess. The detailed review of Barceló and Poschenrieder (2006) came to the conclusion that except for Cd, no visible ultrastructural damage is observed in the organelles of the roots, but the metals rather disturb the polar zonation of the organelles within the cells. Changes in the amyloplasts and their arrangement in root columella cells (Table 26.3) may directly influence root gravitropism and growth direction and seem to be associated with, for example, Al stress–induced root growth defects. Sometimes, alterations in plastid shape were also reported, for example, in Cr<sup>6+</sup> and <sup>99</sup>Tc-treated roots, where amoeboid plastids occurred in the roots (Table 26.3, Vázquez et al. 1987, 1990). Similarly, metal stress altered sugar metabolism and often, changes in the starch contents of plastids have been reported (Table 26.3). The Cd-induced ferritin accumulation in bean root plastids may be related to disturbed Fe homeostasis of these plants (Vázquez et al. 1992). Cu excess–induced the formation of dark globular inclusions of unknown nature in plastids (Panou-Filotheou and Bosabalidis 2004).

Another important, but poorly studied field is the effect of metals on plastid differentiation, on the biosynthesis of the photosynthetic apparatus and on chloroplast biogenesis. These studies are even more important, as seedlings germinating in polluted soil have to cope with metal stress at this level, and seedlings that fail to develop functionally active chloroplasts do not survive. Chloroplast differentiation may proceed directly from proplastids, or in case of agricultural systems, seedlings germinating from seeds buried in the soil often develop etioplasts before reaching the soil surface (reviewed by Solymosi and Schoefs 2008). Therefore, studies related to the impact of excess metals on proplastids, etioplasts or young chloroplasts differentiating from these two plastid types are crucial to understand the molecular interactions of metals with these organelles and also to enhance seedling survival in polluted areas.

Several metals (Cd, Na, K and Hg) influence the etioplast to chloroplast transformation (Table 26.3). These elements induce slight structural alterations in etioplast structure, which are in some respect similar to those observed in chloroplasts (see later, i.e., swelling of intrathylakoidal space, formation of vesicles, regularly spotted bodies indicating possible osmotic stress). Chl biosynthesis and etioplast–chloroplast transition (the reorganization of the inner membrane system and the development of the thylakoids and the photosynthetic apparatus) is impaired under different metal stresses under in vitro conditions with short-term, high-concentration treatments (excess Cd: Ghoshroy and Nadakavukaren 1990; excess Na and K: Abdelkader et al. 2007; excess Hg: Solymosi et al. 2006b). This might indirectly indicate that these processes may be also partially inhibited in the light.

26.3.2 IMPACT OF UNBALANCED METALS ON PHOTOSYNTHESIS

Chloroplasts are key organelles for plant development, growth and biomass production because of their ability to produce sugars via photosynthesis. On the other hand, photosynthesis is a process that requires several metalloproteins containing different HMs (Table 26.2). The different essential metals present in plastids, besides being structural constituents of various molecules, are also important in modulating reactions or cellular processes, or in maintaining the ion homeostasis of the organelles or the cells. For instance, plastids seem to have a role in Ca (Seigneurin-Berny 2000), Cu (Abdel-Ghany 2009), and Fe (Izaguirre-Mayoral and Sinclair 2005, 2009) storage, Fe being often stored in the metabolically inactive form of phytoferritin, which prevents photooxidation reactions caused by “free” metal ions (Briat et al. 1999, Arosio and Levi 2002, Ravet et al. 2009).

As discussed earlier, plants possess different uptake systems to transport essential metals into the plastids (Table 26.1). Part of these transporters can also deliver nonessential elements. However, the knowledge about plastid metal transporters in respect to metal stress is relatively scarce and it is difficult to determine the exact concentration of metals inside chloroplasts. Most of such studies have been done on algae, in which the accumulation of different metals can be easily observed in thylakoid membranes (reviewed by Barceló and Poschenrieder 2006), but they are not relevant for terrestrial plants. Only rough estimations exist, that assume that 1% of total Cd content of plants is probably transported to plastids (reviewed by Siedlecka and Krupa 1999). Some data about metal concentrations in chloroplasts are summarized in Table 26.4. Unfortunately, even if available, often other literature data about plastid metal contents are not comparable, because they are expressed in different and non-interconvertible units or on different basis (e.g., Cd: Ramos et al. 2002; Cu: Baszyński et al. 1978; Zn, Mg, Cu: Kim et al. 2009) or were determined only in thylakoids (e.g., Mn: Lidon and Teixeira 2000a, Lidon et al. 2004) or were measured after different durations of metal treatments (e.g., Ramos et al. 2002).

TABLE 26.4  
Intraplastidial Concentration of Some Essential Metals

| Metal | Concentration within the Plastid  | References                |
|-------|-----------------------------------|---------------------------|
| Ca    | Total: 4–23 mM, free: few $\mu$ M | Johnson et al. (2006)     |
| Cu    | 60 $\mu$ M                        | Joyard and Douce (1976)   |
| Fe    | 0.13 mM                           | Joyard and Douce (1976)   |
| K     | 150 mM                            | Neuhaus and Wagner (2000) |
| Mg    | 5 mM                              | Neuhaus and Wagner (2000) |
| Mn    | 33 $\mu$ M                        | Joyard and Douce (1976)   |
| Zn    | 0.13 mM                           | Joyard and Douce (1976)   |

At the same time, the mode of HM pollution is also important in determining the interactions of the metals with plastids. In the leaves of lettuce seedlings treated with Cd solutions for 16 days through their roots, the lowest intracellular Cd concentration ( $6\text{--}16\text{ }\mu\text{g g}^{-1}\text{ FW}$ , 12%–14% of total Cd content of leaves) was found in the chloroplasts (Ramos et al. 2002). Interestingly, the Cd content of the chloroplasts was  $10\text{--}22\text{ }\mu\text{g g}^{-1}\text{ FW}$  (8%–9% of total leaf Cd content) when lettuce leaves were incubated for 24 h in Cd-containing solutions, a model experiment mimicking airborne metal pollution (Ramos et al. 2002). In both cases, most Cd was accumulated in the apoplasm. These studies indicate that, in case of airborne metals, atmospheric metal deposition may have a stronger influence on plastid metabolism than soil pollution (Ramos et al. 2002).

Developing methods of electron microscopy combined with analytical techniques such as energy dispersive x-ray microanalysis (EDXA), laser microprobe mass analysis (LAMMA), electron energy loss spectroscopy (EELS), Synchrotron x-ray fluorescence (SXRF) microbeam analyses, secondary ion mass spectrometry or cytochemical methods are powerful tools to characterize primary mechanisms of metal toxicity and tolerance on the cellular and molecular level (reviewed by Barceló and Poschenrieder 2006). Similarly, atomic force microscopy, laser scanning optical microscopy using confocal microscopy or multiphoton excitation provide important information about metal stress on the cellular and tissue level. New methods are also under development (e.g., laser-induced breakdown spectroscopy and laser-ablation inductively coupled plasma mass spectrometry—Kaiser et al. 2009). Unfortunately, data about microlocalization of metals in plastids with these imaging methods are relatively scarce, probably because of the low concentrations accumulating in these organelles being under the actual detection limit of these methods. However, direct interaction of the metals with chloroplast membranes and metabolism cannot be excluded even in cases when the amounts of metals were below the detection limit in the plastids. Therefore, we overview some data that reported direct connections between the absence or the presence of metals in plastids, and observed metabolic alterations in vivo.

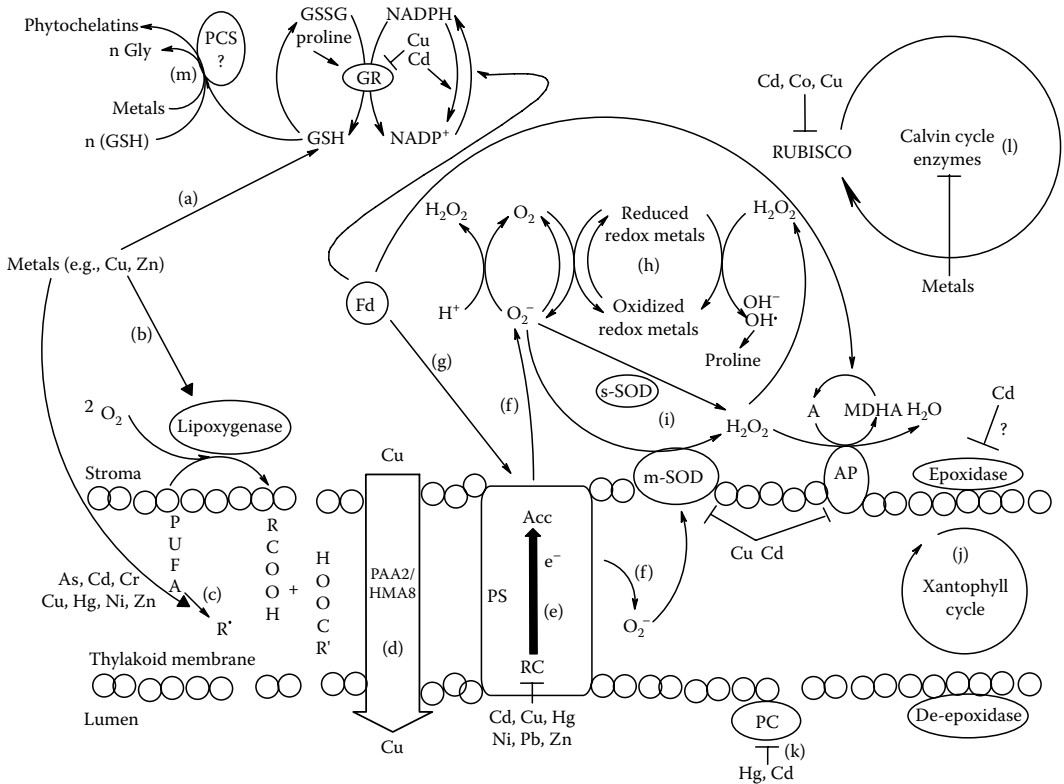
Chl biosynthesis requires several metals; therefore, decreased Chl content is a general symptom of essential-HM deficiency (Cu: Burkhead et al. 2009, Hänsch and Mendel 2009, Yruela 2009; Fe: Puig et al. 2007; Mn: Simpson and Robinson 1984, González and Lynch 1999, Yu et al. 1999, Henriques 2003, 2004; Zn: Singh et al. 2005). There are almost no data about metal concentrations in plastids under HM deficiency. Different estimations indicate that about 80% of foliar Fe is present in the plastids (Terry and Low 1982, Thoiron et al. 1997), and in *Arabidopsis* green tissues, 40% of Fe is found in the thylakoids (reviewed by Briat et al. 2007), so these organelles can be expected to be one of the first targets of Fe-deficiency. Chlorotic leaves of Fe-deficient plants have yellowish color because of decreased Chl content due to impaired Chl biosynthesis and the symptoms appear first in the interveinal areas (Thoiron et al. 1997, Misra et al. 2006, Mahmoudi et al. 2007, Timperio et al. 2007). Some authors have postulated that Fe-deficiency induced chlorosis is related to decreased Fe and Chl concentrations in thylakoid lamellae, and this way to retarded or disturbed thylakoid formation rather than to inhibited Chl biosynthesis per se (Terry and Low 1982). Moreover Fe-deficiency would impact photosystem I (PSI) functioning because of the reduced number of Fe-S clusters (Doan et al. 2003, Duy et al. 2007).

The effect of excess HMs is often also reflected by reduced plant growth and chlorosis. In these cases, often the observed phenotypic alterations could be directly linked with the presence of the excess metals in the chloroplasts. For instance, the toxic effects of Ni on chloroplast structure of cabbage plants (Molas 1997, 2002) are in good agreement with the observed Ni accumulation inside the organelles. The histochemical techniques of Ni localization show that in cabbage plants the important sites for Ni accumulation in the leaf are mesophyll cells located on leaf edges, near the vascular bundles and intervacular bundles affected with chlorosis (Molas 2002). The chloroplast, the cell walls and the nucleus are the most important Ni-accumulation sites at the cellular level.

In rice plants treated with increasing concentrations of Mn, the accumulation of this metal was found in the leaves and thylakoid membranes, which indicates that this element is highly mobile, enters the chloroplasts and accumulates up to a certain level in these organelles (Lidon et al. 2004). Similarly, it has been demonstrated that large part of Mn quantity entering the cytoplasm moves and

binds on the outer side of thylakoid membranes of chloroplasts (González and Lynch 1999, Lidon and Teixeira 2000b, Lidon et al. 2004) affecting their structure and photosynthesis. Moreover leaf chlorosis induced by excess Mn was positively correlated to Mn content of chloroplasts (González and Lynch 1999). Therefore, in this case, the observed ultrastructural symptoms might be at least partially linked to direct interactions of Mn with thylakoid membranes.

Chloroplasts are one of the main sites of Cu accumulation (Maksymiec 1997). Data indicate that under Cu treatment, this ion can enter the cytoplasm and the plastids also by Ca channels especially in younger plants and at the initial stages of the stress (Maksymiec and Baszyński 1999). Cu can bind directly to the thylakoids where it may induce oxidative stress (Figure 26.1, reviewed by



**FIGURE 26.1** Oxidative stress and defense reactions occurring in the chloroplast, when excess of metal is present. (a) Depletion of sulfhydryl groups by metals on reduced glutathione (GSH) regenerated by glutathione reductase (GR) from its oxidized form (GSSG); (b) Activation of lipoygenase by metals; (c) Peroxidation of polyunsaturated fatty acids (PUFA) to saturated fatty acids (RCOOH + R'COOH) in membranous phospholipids; (d) Cu transporter PAA2/HMA8; (e) Electron transfer from the reaction center (RC) to the acceptor (Acc) of PSI or PSII; (f) univalent oxygen reduction by PSII; (g) Electron transfer through ferredoxin (Fd) and NADP<sup>+</sup> (specific to PSI); (h) Fenton and Haber–Weiss reactions: one-electron oxidoreductions performed by redox metals leading to hydroxyl radicals (OH<sup>•</sup>); (i) Spontaneous and/or superoxide dismutase (SOD) catalyzed disproportionation of superoxides (O<sub>2</sub><sup>•−</sup>). Superoxide radicals generated by PSI and PSII are dismutated by the membrane-bound m-SOD, and the formed hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is reduced to water by the thylakoid-bound ascorbate peroxidase (AP). Monodehydroascorbate (MDHA) is reduced back to ascorbate (A) by the photoreduced ferredoxin (Fd). O<sub>2</sub><sup>•−</sup> ions escaped from the thylakoid system are scavenged by a stromal s-SOD; (j) Xanthophyll cycle associated to the enzymes epoxidase and de-epoxidase, preventing superoxide formation at high light intensity; (k) Inactivation of plastocyanin (PC) by nonessential metals after its release from the membrane or the release of its Cu; (l) Inactivation of Calvin cycle enzymes; (m) Chelation of metals by phytochelatin (PCS). → activation by metals; ⊥ inhibition by metals.

Maksymiec 1997). Under excess Cu, the Cu content of leaf chloroplasts showed more than tenfold increase on the protein or Chl content basis (Baszyński et al. 1988), which indicates that this element might directly interact with chloroplast components or might induce oxidative stress directly.

In case of poorly mobile, nonessential metals such as Cr, present in the soil, the ultrastructural changes induced in the plastids and chlorosis are probably due to indirect effects of the metal only, because they appear without substantial increase in the metal concentration in the leaves (e.g., Moustakas et al. 1997, reviewed by Barceló and Poschenrieder 2006).

As Table 26.2 lets it assume, each essential HM has specific functions and is needed in appropriate amounts within chloroplasts. Thanks to efficient ion homeostasis, plant development and functioning may be optimal also under moderate HM stress, but large metal unbalance is a disaster for plastids both on the structural and metabolic levels.

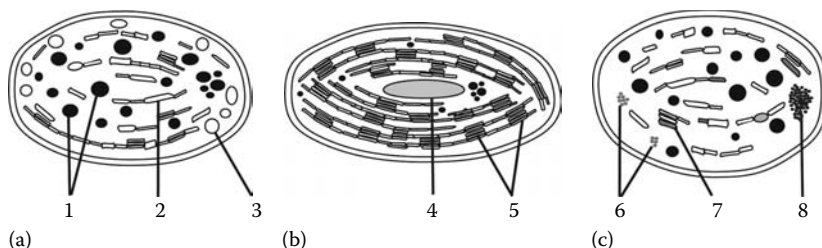
### 26.3.2.1 Ultrastructural Alterations in Chloroplasts

As some HMs are crucial for the functioning of several proteins and structural components of chloroplasts (Table 26.2), it is not surprising that too low concentrations of HMs may cause chlorosis, functional alterations of photosynthesis and other ultrastructure. Before discussing the effects of toxic concentrations of metals on chloroplast structure, we briefly review the symptoms of essential-metal deficiency.

Ultrastructural alterations are easy to be observed, but are more complicated to be interpreted on the metabolic level. Since unbalanced metal concentrations can be the result of several phenomena (including competition of essential and nonessential metals for transport systems in plants and this way to indirect effects), and to interaction of different metals with each other on several levels, including metabolic levels, the most reliable data about the direct ultrastructural effects of essential-metal deficiency are those that were obtained in nutrient transporter mutants impaired in chloroplast metal uptake or homeostasis (Henriques et al. 2002, Song et al. 2004, Duy et al. 2007). The disturbances in thylakoid biosynthesis can be in this case directly related to nutrient deficiency, outlining the importance of essential metals in plastid differentiation and in the maintenance of the active photosynthetic apparatus.

Some recently characterized knockout mutants impaired in Fe and Zn uptake (*irt1*) or in their transport into the plastids (*pic1*) show several ultrastructural alterations at the chloroplast level. *Irt1* mutants have reduced thylakoid system and stacking (maximum 2–3 thylakoids per grana), smaller starch grains and increased number of plastoglobuli (Henriques et al. 2002). *Pic1* knockouts have unchanged Fe level, but changed ion homeostasis, Cu content, and their internal membrane system is significantly reduced (Duy et al. 2007). No grana, just simple and parallelly arranged thylakoids or even no thylakoid characterize the plastids. Vesicle formation and increased ferritin content are also characteristic in these mutants (Duy et al. 2007).

There are several other data in the literature that describe the ultrastructural alterations of chloroplasts of plants grown under essential-HM deficient conditions (Figure 26.2, Table 26.5).



**FIGURE 26.2** Scheme summarizing the most important ultrastructural alterations of chloroplasts (b) induced by essential metal deficiency (a) or by excess of essential or nonessential metals (c). Note the swelling of the organelle and the alterations of the different structural elements of chloroplasts (1: plastoglobuli, 2: swollen intrathylakoidal space, 3: peripheral vesicles, 4: starch grains, 5: grana, 6: regularly spotted bodies, 7: dark deposits on the thylakoid surface 8: ferritin clusters).



**TABLE 26.5**

**Chloroplast Alterations of Crop Plants Caused by Essential Metal Deficiency (on White Background) or by Excess of Heavy Metals (HMs) (on Gray Background)**

| HMs | C<br>Number | C<br>Size | Disturbed<br>C Shape | DE | TD | ST | Size<br>of PG | Number<br>of PG | S  | V | F | SB | DD | References                                                                                                                                                                                       |
|-----|-------------|-----------|----------------------|----|----|----|---------------|-----------------|----|---|---|----|----|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cd  | ↓           |           | +                    | +  | +  | +  | ↑             | ↑               | ↑  |   |   | +  |    | Baszyński et al. (1980), Barceló et al. (1988), Ouzounidou et al. (1997), Baryla et al. (2001), McCarthy et al. (2001), Sandalio et al. (2001), Carrier et al. (2003), and Djebali et al. (2005) |
| Cr  |             |           | +                    |    | +  | +  |               |                 |    |   |   |    |    | Vázquez et al. (1987)                                                                                                                                                                            |
| Cu  |             |           |                      |    | +  | +  | ↑             | ↑               |    |   |   |    |    | Baszyński et al. (1978) and Henriques (1989)                                                                                                                                                     |
| Cu  | ↓↑          | ↓         | +                    | +  | +  | +  | ↑             | ↑↓              | ↓  |   |   | +  |    | Baszyński et al. (1988), Eleftheriou and Karataglis (1989), Maksymiec et al. (1995), Ciscato et al. (1997), Quartacci et al. (2000), Panou-Filothou et al. (2001), and Bernal et al. (2006)      |
| Fe  |             | ↓         |                      |    | +  | +  |               | ↑               | ↓↑ | + |   |    |    | Platt-Aloia et al. (1983), Ji et al. (1984), Thoiron et al. (1997), Briat et al. (1999), and Henriques (2003)                                                                                    |
| Fe  |             |           |                      |    |    |    |               |                 |    |   | + |    |    | Izaguirre-Mayoral and Sinclair (2005)                                                                                                                                                            |
| Mn  | ↓           | ↓         |                      |    | +  | +  |               | ↑               | ↓  | + |   |    |    | Mercer et al. (1962), Possingham et al. (1964), Weiland et al. (1975), Henriques (2003, 2004), Izaguirre-Mayoral and Sinclair (2005), and Papadakis et al. (2007)                                |
| Mn  |             | ↑         | +                    |    | +  | +  | ↑             | ↑               | ↑  |   |   |    | +  | Lidon and Teixeira (2000a), McQuattie and Schier (2000), and Papadakis et al. (2007)                                                                                                             |
| Ni  |             |           |                      |    | +  | +  | ↑             |                 | ↑  |   |   |    |    | Molas (1997, 2002)                                                                                                                                                                               |
| Pb  |             |           |                      | +  | +  |    | ↑             | ↑               | ↑  |   |   | +  | +  | Woźny et al. (1995), Weryszko-Chmielewska and Chwil (2005),                                                                                                                                      |
| Tc  |             |           | +                    | +  |    |    |               |                 | ↓  |   |   |    |    | Vázquez et al. (1990)                                                                                                                                                                            |
| Zn  |             |           | +                    | +  | +  | +  |               | ↑               |    |   |   |    |    | Thomson and Weier (1962), Henriques (2001), and Chen et al. (2008)                                                                                                                               |
| Zn  |             |           |                      |    | +  | +  | ↑             | ↑               | ↓  |   |   |    |    | Doncheva et al. (2001)                                                                                                                                                                           |

*Notes:* Plus signs indicate that the alteration was described by authors, upward (↑) and downward (↓) arrows indicate increase and decrease, respectively. Abbreviations: C, chloroplast; DD, dark deposits at thylakoids; DE, disruption of the envelope; F, ferritin; PG, plastoglobuli; S, starch content; SB, regularly spotted body; ST, swollen intrathylakoidal space; TD, thylakoid disorders; V, unusual peripheral vesicles.

Chloroplasts are often swollen in such plants (Zn deficiency: Chen et al. 2008), or sometimes have smaller size than in plants grown under optimal metal concentrations (Fe deficiency: Platt-Aloia et al. 1983; Mn deficiency: Papadakis et al. 2007). Rarely, the disruption of the plastid envelope has been observed in such plants (Zn-deficient rice: Chen et al. 2008). Abnormal chloroplasts with almost no grana and disrupted or disorganized internal membranes (Cu deficiency: Thoirion et al. 1997, Briat et al. 1999, Henriques 2003; Mn deficiency: Possingham et al. 1964, Weiland et al. 1975, Izaguirre-Mayoral and Sinclair 2005; Zn deficiency: Thomson and Weier 1962, Henriques 2001, Chen et al. 2008), the swelling (dilatation) of the intrathylakoidal space (Cu deficiency: Baszyński et al. 1988, Henriques 1989; Fe deficiency: Thoirion et al. 1997; Mn deficiency: Weiland et al. 1975, Izaguirre-Mayoral and Sinclair 2005, Papadakis et al. 2007; Zn deficiency: Chen et al. 2008), the appearance of clusters of unusual, peripheral vesicles (Fe deficiency: Platt-Aloia et al. 1983, Briat et al. 1999; Mn deficiency: Mercer et al. 1962, Possingham et al. 1964), increased number (and size) of plastoglobuli (Cu deficiency: Henriques 1989; Fe deficiency: Ji et al. 1984, Henriques 2003; Mn deficiency: Weiland et al. 1975, Izaguirre-Mayoral and Sinclair 2005; Zn deficiency: Chen et al. 2008) are usual symptoms of metal deficiency. The starch contents of plastids also vary with different essential-HM availability; however, these changes are not consistent, i.e., Fe deficiency causes decreased starch content in apple chloroplasts (Ji et al. 1984), while starch content increases in chloroplasts of Fe-deficient pecan (Henriques 2003). Decreased starch size and content is observed in plastids of Mn-deficient lemon (Papadakis et al. 2007) and soybean (Weiland et al. 1975, Izaguirre-Mayoral and Sinclair 2005).

Several data are available about the ultrastructural alterations caused by the excess of various HMs added in various treatments, under various experimental conditions and in various plant species. However, interestingly the symptoms are quite similar (Table 26.5). Most studies have been conducted under laboratory conditions (e.g., hydroponic cultures, etc.), but sometimes the plastid ultrastructure of plants grown in naturally contaminated soils has been also described (wheat plants grown in Cu-contaminated soils: Eleftheriou and Karataglis 1989). These plants contain a reduced number and a reduced size of chloroplasts, less developed plastid inner membrane system; they also have impaired grana formation, decreased starch content and decreased number of plastoglobuli (Eleftheriou and Karataglis 1989).

In some cases, different steps of plastid ultrastructural alterations were distinguished as a function of increasing concentrations and/or duration of metal exposure (e.g., Na: Rahman et al. 2000; Zn: Doncheva et al. 2001). In this latter case, the first symptoms of Zn stress observed at lowest HM concentrations is the disintegration of stromal thylakoids, and the reduction of grana number, swollen thylakoids (i.e., the swelling of the intrathylakoidal space), decreased starch content, increase in size and number of plastoglobuli appear subsequently with increasing Zn concentrations (Doncheva et al. 2001).

In the leaves of plants grown in experimental conditions with excess of HMs, the number of chloroplasts may decrease (Table 26.5), which is probably due to metal interference with chloroplast replication (reviewed by Kucera et al. 2008). Besides this phenomenon, damage to chloroplasts is the most frequently observed ultrastructural effect of toxic metals in leaves. The inhibition of normal plastid development in HM-treated plants may be indicated by the appearance of amoeboid plastids observed occasionally (Vázquez et al. 1990) and is further supported by the fact that, in general, chloroplasts of young leaves are more affected by metal stress than old leaves (e.g., Maksymiec et al. 1995, Skórzyńska-Polit and Baszyński 1997, Maksymiec and Baszyński 1999). However, contradictory data have been also published (Barceló et al. 1988), and these observations might not be generalized, they also depend on the developmental stage at which metal pollution occurs. The most common ultrastructural symptoms are the swelling of the organelle, distortion of thylakoids leading to the loss of the parallel arrangement of the thylakoid membranes, reduction or increase of the thylakoid surface area, and swelling of the intrathylakoidal space (Table 26.5, reviewed by Barceló and Poschenrieder 2006). There are contradictory data about the changes in the starch content upon HM excess, which may increase or decrease in the plastids (Table 26.5). Often, the

senescence of the chloroplasts, i.e., the chloroplast-gerontoplast conversion, is induced by the excess of metals (Table 26.5), which is not only indicated by the degeneration of grana and disorders in the thylakoid system but also by the increase in the number and size of plastoglobuli. These symptoms may be a result of metal-induced alteration of the hormone balance (e.g., Cd: Vassilev et al. 2004, Rodríguez-Serrano et al. 2009, reviewed by Maksymiec 1997, for more details about all metals, see Fodor 2002, Maksymiec 2007) leading to enhanced senescence. Most studies have dealt with plastid ultrastructure in leaves, however, some data indicate that chloroplasts in the green stems are also similarly damaged by excess of metals (Barceló et al. 1988).

The careful comparison of the ultrastructural effects induced by essential-HM deficiency and excess of HMs (Figure 26.2, Table 26.5) indicates that besides several similar structural alterations, there are specific differences. Regularly spotted bodies, appearance of dark electron dense deposits at grana surfaces and ferritin were only related to the presence of excess HMs and might therefore be specific for this type of stress, while unusual peripheral vesicles appear under metal-deficient conditions (Figure 26.2, Table 26.5). Disruption of the plastid envelope was more often observed in plastids of plants treated with excess HMs.

These results further outline the complicated interrelations and the difficulties in interpreting the effects of HMs at the molecular and cellular level. The observed effects have been mostly interpreted as the excess or deficiency of HMs on membranes or osmotic disturbances. However, it is unclear to what extent these ultrastructural effects are due to direct toxicity of the metal ions in the chloroplast, to metal-induced membrane disturbances, to metal-induced enhancement of ROS, to osmotic problems, or to consequences of metal-induced deficiency of essential nutrients. In the following sections we briefly review the possible reasons of the observed ultrastructural alterations.

### 26.3.2.2 Molecular and Metabolic Alterations in Chloroplasts under Heavy-Metal Deficiency

Essential HMs are needed for normal plastid ultrastructure, homeostasis, and functioning (i.e., they represent functional components of the thylakoids and the stroma, take part in plastid protein synthesis, DNA replication, ... etc., Table 26.1), therefore, it is not surprising that their structure is strongly influenced in plants grown under nutrient-deficient conditions (Figure 26.2, Table 26.5). Nutrient-deficiency symptoms are usually expressed as reduced growth, biomass, and physiological functions. Chl biosynthesis requires several metals (Table 26.2); therefore decreased Chl content is a general symptom of essential-HM deficiency (Cu: Burkhead et al. 2009, Hänsch and Mendel 2009, Yruela 2009; Fe: Puig et al. 2007; Mn: Simpson and Robinson 1984, González and Lynch 1999, Yu et al. 1999, Henriques 2003, 2004; Zn: Singh et al. 2005). Not only because of the decreased Chl content, but due to direct interactions with photosynthetic reactions, the rate of CO<sub>2</sub> fixation and biomass also decrease (e.g., Zn deficiency: Srivastava et al. 1997; Cu-deficiency: Droppa et al. 1984, Maksymiec 1997).

Oxidative stress is also one of the most important components of mineral-nutrient-deficiency stresses (e.g., Zn, Mn, Fe, Cu, B, Mg, and K) (Yu et al. 1999). This is not surprising as some micronutrients, such as Zn, Fe, and Cu are important components of ROS scavenging systems of plastids (Cu/Zn and Fe superoxide dismutases, Table 26.2), therefore, plants deficient in these metals may exhibit symptoms of oxidative stress. Below, we briefly overview some metabolic processes impacted by the deficiency of the most important essential HMs.

Fe-deficiency affects not only Chl biosynthesis (Duy et al. 2007, reviewed by Myśliwa-Kurdziel and Strzałka 2002), but the functioning of the photosynthetic electron chain, the Calvin cycle (Siedlecka and Krupa 1996, reviewed by Myśliwa-Kurdziel and Strzałka 2002), plastid protein import (Duy et al. 2007), enzymes of chloroplast-localized nitrogen fixation machinery (Briat and Vert 2004), sulfur assimilation, siroheme biosynthesis, amino acid metabolism (Duy et al. 2007), and Fe-involving antioxidative response (Allen 1995) (Table 26.2). The different molecular alterations of the photosynthetic apparatus induced by Fe-deficiency (Sárvári 2005, Briat et al. 2007) and the adaptation mechanisms of photosynthesis under Fe starvation (Sharma 2007) are summarized

elsewhere. Some recently characterized mutants impaired in Fe uptake (*irt1*) or in its transport into the plastids (*pic1*) have similar ultrastructural damage as reported in Fe-deficient plants (Figure 26.2) (Henriques et al. 2002, Duy et al. 2007). The disturbances in thylakoid biosynthesis and plastid differentiation can be in this case directly related to nutrient deficiency, outlining the importance of essential metals in plastid differentiation and in the maintenance of the active photosynthetic apparatus.

Zn deficiency also leads to chlorosis and decreased photosynthetic activity in leaves (Cakmak 2000, Henriques 2001, Wang and Jin 2005, Chen et al. 2008). Leaves become then light sensitive. The leaves of Zn-deficient sugar beet plants have disorganized chloroplasts with senescence process leading to cell death and, ultimately, to necrotic blade lesions, thus reducing the photosynthetically-active leaf area, which explains the lower CO<sub>2</sub> fixing capacity and decreased biomass of Zn-deficient plants (Henriques 2001). The observed symptoms are probably associated with oxidative stress (for a review see Cakmak 2000) and decreased antioxidant enzyme levels (e.g., Cu/Zn-superoxide dismutase = Cu/Zn-SOD) in Zn-deficient leaves (Wang and Jin 2005, Chen et al. 2008, reviewed by Cakmak 2000, Rengel 2006). In Zn-deficient chickpea plants, disturbances in stomatal conductance and water status were also observed (Khan et al. 2004). The reduced growth and productivity of Zn-deficient plants are often associated with decreased levels of indole-3-acetic acid (reviewed by Cakmak 2000). Zn deficiency also alters membrane lipid composition and fatty acid saturation and leads to lipid peroxidation. Zn deficiency induces a decrease in the activity of carbonic anhydrase, which catalyzes the reversible reaction of CO<sub>2</sub> hydration and is therefore accompanied by reduced photosynthetic rates (reviewed by Rengel 2006).

Cu is a crucial plastid component involved in photosynthetic electron transport (plastocyanin) but is also indispensable for photosystem II (PSII) and light-harvesting complex II (LHC II) and for the functioning of the antioxidant enzyme, Cu/Zn-SOD (reviewed by Maksymiec 1997). Fifty percent of total plastidic Cu is found in plastocyanin (reviewed by Hänsch and Mendel 2009). Therefore, it is not surprising that photosynthetic functions are highly impaired under Cu-deficiency. Decreased plastocyanin, Chl, and carotenoid contents observed in Cu-deficient leaves can be responsible for the lower rates of photosynthesis (Baszyński et al. 1978, Henriques 1989, Shikanai et al. 2003, reviewed by Burkhead et al. 2009). PSI activity and cyclic photophosphorylation (Baszyński et al. 1978) as well as PSII activity are also targets of Cu-deficiency (reviewed by Myśliwa-Kurdziel and Strzałka 2002). Cu deficiency is often accompanied with oxidative stress probably due to decreased Cu/Zn-SOD levels (reviewed by Rengel 2006). The lipid composition of Cu-deficient chloroplasts is altered (reviewed by Barón et al. 1995). The galactolipid content decreases and the fatty acid unsaturation levels are also altered in the different lipid fractions (they increase in galactolipids). A secondary effect of Cu deficiency can be insufficient water transport caused by a decrease in cell wall formation and lignification in several tissues, including xylem tissue (for a review, see Burkhead et al. 2009).

Mn-deficiency symptoms include reduced growth, pale young leaves that subsequently develop interveinal chlorosis, and ultimately small necrotic spots (Yu et al. 1999, Henriques 2003, Henriques 2004). Mn deficiency also induces oxidative stress that could be partially prevented in transgenic tobacco plants overexpressing Mn-SOD in chloroplasts (Yu et al. 1999). Mn-deficient plants show the loss of most, but not all, functional PSII reaction centers in grana, with no alteration in light-harvesting complex of PSI, which is linked to the disruption of the oxygen-evolving complex (Simpson and Robinson 1984). However, upon Mn resupply, the leaves become able again to control Mn levels after 2 days. Mn deficiency depresses leaf photosynthetic capacity primarily by reducing the number of PSII units in spinach leaves (e.g., Simpson and Robinson 1984). Recent investigations in pecan leaves have shown that the reduced number of PSII units per leaf area unit is achieved by decreasing the number of chloroplasts, but not the number of PSII per individual chloroplast, and that the remaining PSII possess photochemical abilities similar to those of control leaves (Henriques 2003, 2004), their Mn content being similar to that of chloroplasts isolated from control plants (Henriques 2004).

### 26.3.2.3 Molecular and Metabolic Alterations in Chloroplasts under Heavy-Metal Excess

Several visible symptoms have been observed in plants grown in the presence of excess essential or nonessential HMs. Leaf expansion is inhibited, leaf tissues can become deformed and chlorosis often occurs (e.g., Cd: Djebali et al. 2005, Ebbs and Uchil 2008, Ben Ghnaya et al. 2009; Cr: Vázquez et al. 1987; Cu excess: Baszyński et al. 1988, Ciscato et al. 1997, Panou-Filothéou et al. 2001; Mn excess: González and Lynch 1999, reviewed by Barceló and Poschenrieder 2006; Pb: Woźny et al. 1995; Zn excess: Doncheva et al. 2001, Ebbs and Uchil 2008, Wang et al. 2009). The inhibitory effect of excess metals on Chl biosynthesis (Myśliwa-Kurdziel and Strzałka 2002) and on photosynthesis (Fodor 2002, Myśliwa-Kurdziel et al. 2004, Sárvári 2005, Briat et al. 2007) is reviewed elsewhere in more detail. In this section, we briefly summarize the different direct and indirect effects of metals on plastid metabolism with emphasis on metal-specific effects and observations.

Clearly, the thylakoid membranes and plastids are much influenced by metal excess (Figure 26.2, Table 26.5); however, it is not clear if they are really the primary targets of metal stress—and therefore, their alteration is responsible for the observed decrease in photosynthetic activity—or if they simply reflect the observed dramatic alterations of plastid metabolism. There are almost no data about the actual in organello concentration of excess metals, and therefore on the direct nature of their interactions. Furthermore several observed symptoms seem to be nonspecific. First, we briefly overview some examples for direct interactions of HMs with different plastid components (proteins, pigments, cofactors, and lipids), then we summarize data about metal-excess induced oxidative stress in plastids and the possible osmotic disturbances observed under excess metals. Finally, the essential metal (Fe, Cu, Zn, Mn)-deficiency induced by the excess of another HM is also discussed.

#### 26.3.2.3.1 Molecules Impacted Directly by Metals

Direct interactions of metals with chloroplast metabolism could be often only studied in vitro at relatively high concentrations that are unlikely to occur in the organelles under natural conditions. These effects are summarized below.

**26.3.2.3.1.1 Pigments and Other Small Plastid Metabolites** Excess HMs may directly interact with pigments and may replace Mg in Chl in vivo or in vitro (Cd, Cu, Hg, Ni, Pb, Zn) (Figure 26.1) (Küpper et al. 1996, 1998, 2002, 2003). At high concentrations, different nonessential metals can also destabilize pigment precursors and may not substitute Mg, but induce its loss from the porphyrin ring (e.g., protochlorophyllide: Solymosi et al. 2004). There is much less data about the direct interaction of excess HMs with carotenoids. In general, carotenoid levels seem to be affected indirectly by HM stress as these molecules have an important role in scavenging ROS induced by HMs (see below). In diatoms, the xanthophyll cycle was shown to be altered by Cd (Bertrand et al. 2001).

The observed decreased photosynthetic activity and disturbed plastid metabolism of HM-treated plants may be related to direct oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) by nonessential HMs (e.g., Cd: Figure 26.1, Böddi et al. 1995, reviewed by Pál et al. 2007; Hg: Lenti et al. 2002, Solymosi et al. 2004, Solymosi et al. 2006b), which might result in the inhibition of the activity of enzymes or metabolic processes that use NADPH as a hydrogen donor. Nonessential HMs have been also reported to cause high ATP content, and to change gene expression through DNA hypomethylation and DNA damage (reviewed by Poirier et al. 2008). This way, their carcinogenic effect has also been reported (e.g., Monteiro et al. 2009). Hg-induced inhibition of photosynthesis occurs probably by inducing a severe loss of adenylate pool and decreasing thus the rate of cyclic and non-cyclic photophosphorylation. Hg also decreases PSII associated reactions, O<sub>2</sub> evolution and CO<sub>2</sub> fixation, probably due to the retardation of all ATP-dependent processes (reviewed by Romanowska 2002).

**26.3.2.3.1.2 Lipids** The observed changes in plastid and chloroplast membrane structure (Figures 26.1 and 26.2, Tables 26.3 and 26.5) might be due, at least partially, to membrane lipid

alterations in metal-exposed plants (reviewed by Devi and Prasad 2006). Nonessential HMs influence the lipid composition, the saturation, or even the chain length of the fatty acids of membrane lipids. Several metals (e.g., Cd: Skórzyńska-Polit et al. 1998, Jemal et al. 2000, Nouairi et al. 2006; Pb: Stefanov et al. 1995) but also excess micronutrients (e.g., Cu: Maksymiec et al. 1992, Quartacci et al. 2000; Mn: Lidon et al. 2004) decrease the monogalactosyl-diacylglycerol (MGDG) content of thylakoid membranes. This change is explained by increased galactolipase activity (Skórzyńska and Baszyński 1993, Stefanov et al. 1995). Other lipid fractions have been also affected, but as MGDG and digalactosyl-diacylglycerol (DGDG) ratios influence membrane curvature, the changes in their ratios can explain the observed alterations in grana stacking and/or grana to stroma thylakoid ratios under metal stress (Table 26.5, Figure 26.2). Similarly, the swollen intrathylakoidal space has been related to decreasing MGDG levels caused by increased galactolipase activity (Skórzyńska et al. 1991). MGDG is also required for proper functioning of photosynthesis (PSII complexes); therefore changes in this lipid fraction may have a detrimental effect on thylakoid functions and the total photosynthetic efficiency of plants. In addition, Ag, Cu, Pb, and Hg inhibit the plastidial phosphatidylcholine synthesis (Akermoun et al. 2002).

The amount of highly unsaturated fatty acids (especially 18:2 and 18:3) has been shown to increase after Cd (maize: Pál et al. 2007), Cu (spinach: Maksymiec et al. 1994), and Pb (spinach: Stefanov et al. 1995) treatment. However, contradictory data indicating lower degree of fatty acid unsaturation have been also reported in other plants treated with Cd (pepper: Jemal et al. 2000; tomato: Djebali et al. 2005; *Brassica napus*: Nouairi et al. 2006, Ben Ammar et al. 2007).

Increased fatty acid desaturation as well as decreased MGDG content can change membrane fluidity (Quartacci et al. 2000), which in turn leads to altered membrane physiological functions; it particularly influences the ionic permeability of the thylakoid membranes (for a review, Sandalio et al. 2001, see Devi and Prasad 2006). Different membrane permeabilities of the thylakoids might also explain the observed swelling of the intrathylakoidal space.

Membrane injuries of metal-treated plants (e.g., disruption of the envelopes, Table 26.5) are often related to increased peroxidation of membrane lipids caused by highly toxic free radicals (ROS) (reviewed by Devi and Prasad 2006, Maksymiec 2007). As, Cr, Cd, Cu, Hg, Ni, and Zn have all been shown to induce lipid peroxidation (Figure 26.1c) (e.g., Cd: Djebali et al. 2005, Skórzyńska-Polit and Krupa 2006; Hg: Cho and Park 2000) as well as several of the Fe and Cu compounds that can catalyze the Haber–Weiss and Fenton reactions (Babu et al. 2001). Cd directly affects the lipid structure around LHCII, leading to lipid peroxidation and the release of several pigment–protein complexes, oxygen-evolving complex (OEC) and plastocyanin (Figure 26.1k), then blocking further electron transport processes (for reviews, see Siedlecka and Krupa 1999, Pál et al. 2006). All these observations indicate that potentially toxic metals enter the plastids and can influence directly the different plastid components and then plastid metabolism.

**26.3.2.3.1.3 Proteins** One of the most often–reported direct toxic effects of metals on plastid proteins is attributed to the ability of some metals to bind to sulfhydryl-, histidyl-, and carboxyl-groups of proteins or enzymes, inducing therefore conformational changes resulting often in protein inactivation or disturbed function (Cd, Pb, Hg: Vallee and Ulmer 1972, Lenti et al. 2002, Solymosi et al. 2004; Cu: Maksymiec 1997, Yruela 2009).

Another possibility is that metals in excess may substitute essential metals in catalytic sites of enzymes (Figure 26.1). For instance, Hg can substitute Cu in plastocyanin (Radmer and Kok 1974), Co replaces Mg in the ribulose biphosphate carboxylase oxygenase (RUBISCO), or Zn in transcription factors (reviewed by Poirier et al. 2008), and Cd replaces Ca in PSII reaction centre, causing the inhibition of PSII photoactivation (Faller et al. 2005, reviewed by Kucera et al. 2008). Cd can replace Zn and Ca in metalloenzymes (reviewed by Clemens et al. 2009). The substitution of Mn by Zn or Cd leads to the inactivation of OEC, and as a consequence, electron donation to PSII is inhibited (for reviews, see Bertrand and Poirier 2005, Pál et al. 2006, Kucera et al. 2008). Induced changes in the arrangement and structure of LHCII decrease the efficiency of excitation energy



capture by PSII and reduce the rate of photosynthetic oxygen evolution. Photophosphorylation rates decrease due to PSII dysfunctioning, without evidence for a direct inhibition of the ATP-synthase by the metal (reviewed by Kucera et al. 2008).

Hg ions can directly interact with some sites in the photosynthetic electron transport chain situated in D1 and D2 proteins, with the Mn cluster in the oxygen-evolving complex (reviewed by Romanowska 2002). Excess Cu inhibits several polypeptides of PSII and PSI (e.g., Lidon and Henriques 1993, Maksymiec et al. 1994). A primary site of Cu inhibition was identified on the antenna Chl *a* molecules of PSII (Lidon et al. 1993).

The enzymes RUBISCO, phosphoenolpyruvate carboxylase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and nitrate reductase have been described as sensitive to excess metal concentrations (Figure 26.21) (reviewed by Siedlecka and Krupa 1999, Romanowska 2002, Myśliwa-Kurdziel et al. 2004, Pál et al. 2006, Kucera et al. 2008).

One possible explanation of the metal-induced chlorosis is that excess metals directly interact with enzymes of pigment biosynthesis. Other possibilities involve enhanced pigment degradation (partially due to oxidative stress), direct interaction with pigment precursors or cofactors required for the process, or metal-induced Fe- or Mg-deficiency as these essential metals have key roles in the biosynthetic processes (reviewed by Myśliwa-Kurdziel and Strzałka 2002). Preferential loss of pigments (e.g., Chl *b* in case of Zn and Cd stress—Ebbs and Uchil 2008) and decreased levels of carotenoids (e.g., excess Cu: Baszyński et al. 1988) are often reported under metal stress and can lead to impaired photosynthetic activities.  $\text{Cr}^{6+}$ ,  $\text{Fe}^{3+}$ , and  $\text{Hg}^{2+}$  have been shown to directly inhibit one of the key enzymes of Chl biosynthesis, NADPH: protochlorophyllide oxidoreductase, in vitro (Lenti et al. 2002, Solymosi et al. 2004, Myśliwa-Kurdziel and Strzałka 2005). Cd also inhibits Chl biosynthesis directly through ALA dehydratase and protochlorophyllide reductase (in vitro: Böddi et al. 1995, Myśliwa-Kurdziel and Strzałka 2005, for reviews, see Myśliwa-Kurdziel and Strzałka 2002, Poirier and Bertrand 2005). However, other scientists have found that neither the synthesis nor the photoreduction of protochlorophyllide is influenced by Cd treatment in greening barley leaves (Horváth et al. 1996).

#### 26.3.2.3.2 Metal-Induced Oxidative Burst

HM ions block the electron flow in PSII, leading to the formation of excited triplet Chl ( $^3\text{Chl}^*$ ), which can react with an oxygen molecule with triplet electronic configuration and by this way induces the formation of singlet oxygen. Oxidative stress leads to an imbalance in the regeneration and removal of ROS, including singlet oxygen ( $^1\text{O}_2$ ), superoxide radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and the most damaging and reactive hydroxyl radical ( $\text{OH}^*$ ), which can lead to further lipid peroxidation and can damage membranes, proteins, and nucleic acids, leading to altered membrane fluidity, loss of enzyme function, and genomic changes, respectively (reviewed by Kucera et al. 2008). Induction of free radicals and ROS by metals is very well documented and might be responsible for membrane injuries and some of the ultrastructural changes observed under metal stress (e.g., Babu et al. 2001, Zhang et al. 2005, Rodríguez-Serrano et al. 2009, reviewed by Sharma and Dietz 2008). However, some elements are considered to be redox-active metals (Cu, Fe) and can therefore directly elicit ROS generation (Gallego et al. 1996, Drakiewicz et al. 2004), while others induce it only indirectly (Cd: Gallego et al. 1996, Pál et al. 2007; Cr and Cu: Yruela 2009; Hg: Cho and Park 2000; Mn: Lidon and Teixeira 2000a,b; Ni: Chen et al. 2009; Zn: Panda et al. 2003, Kawachi et al. 2009, reviewed by Sharma and Dietz 2008).

The different antioxidant enzyme systems (such as catalase localized into peroxisomes and plastids, SOD in the cytosol, mitochondria and plastids, peroxidases in vacuoles, cell walls and cytosol, and the ascorbate–glutathion cycle in several plant cell compartments) as well as carotenoids (and particularly the xanthophyll cycle) may protect the plants under oxidative stress and can therefore be responsible for metal tolerance or can indicate metal stress (e.g., Drakiewicz et al. 2004, reviewed by Kucera et al. 2008). Ascorbate takes part in growth processes, electron transport, photoprotection, regulation of photosynthesis, and preservation of the enzymatic activities that contain

prosthetic transition metal ions. Similarly, carotenoids that can quench the oxidizing ROS and the triplet state of Chl are often affected by metal stress (reviewed by Kucera et al. 2008). The carotenoid content of metal-stressed leaves also changes, and particularly the xanthophyll cycle pigments that belong to a protection system for the photosynthetic apparatus from the nonphotochemical quenching of excited triplet Chl and from ROS (reviewed by Kucera et al. 2008). Therefore, high concentrations of antioxidative enzymes, together with enhanced pigment synthesis may allow a plant to completely overcome the harmful action of a toxic metal and to show normal healthy growth. However, the plant still accumulates fairly high amounts of the metal, a feature that can be useful for phytoremediation but dangerous in case of edible crops.

#### 26.3.2.3.3 *Metal-Induced Disturbances in Plastid Water and/or Ion Homeostasis*

It is well documented that several HMs influence gas exchange and transpiration of plants (by influencing root hair formation, membrane permeability, number and diameter of vascular bundles, stomatal conductance changes, and by inducing the closure of stomata) and therefore cause disturbances in respiration, in CO<sub>2</sub> fixation, in the water and nutrient status of plants (e.g., Cd: Shi and Cai 2008, Nedjimi and Daoud 2009, Sayyad et al. 2009; Cr: Vázquez et al. 1987; Cu: Sayyad et al. 2009; Hg: Martínez-Ballesta et al. 2003; Mn: Lidon et al. 2004; Pb: Sayyad et al. 2009; Zn: Sayyad et al. 2009).

The inhibitory effect of metals on the dark phase of photosynthesis is also a complex phenomenon. Increasing stomatal and mesophyll resistance leads to reduced CO<sub>2</sub> uptake, because of a reduced number of stomata or stomatal closing (e.g., Moustakas et al. 1996, Shi and Cai 2008), which might directly inhibit crop production. Cr<sup>6+</sup> treatment in bean has shown that this metal delayed or inhibited the differentiation of stomata on leaves (Vázquez et al. 1987). Similarly, under excess Ni, fewer stomata developed in cabbage leaves and many stomata were defective (Molas 1997). Decreased respiration and transpiration definitely alter the water and nutrient status of the plants. These data taken together, indicate that it is not surprising that sometimes the symptoms of metal excess—even at the plastid level—resemble those observed under osmotic disturbances or changes in water relations.

Some changes in the chloroplast shape and size (Figure 26.2, Table 26.5) or the disruption of the plastid envelope may indicate some osmotic disturbances of the membranes or changes in the envelope membrane permeability (Moustakas et al. 1997). In Cd- or Cu-stressed plants, chloroplasts often exhibited small, regularly spotted bodies, that were described as “pseudocrystalline bodies” (Cu: Ciscato et al. 1997) or “microtubule-like” structures (Cd: Ouzounidou et al. 1997) in wheat leaves. Similar structures have also been described in etioplasts of other species (barley: Wellburn et al. 1982, Wellburn 1984; wheat: Artus et al. 1990) and in greening plastids of Pb-treated barley leaves (Woźny et al. 1995) or salt-stressed etiolated wheat leaves (Abdelkader et al. 2007) or wheat leaves grown in unusual environments (Solymosi, unpublished results). These spotted bodies might resemble the so-called prothylakoid bodies of unstressed plants (Wellburn 1984) or the so-called stromacenters formed in leaves dehydrated by plasmolysis, wilting or grown in windy areas (Gunning 1965, Gunning et al. 1968, Gunning and Steer 1975) and showing therefore some symptoms of water and osmotic imbalance.

It should be mentioned that direct osmotic effects are probable to occur in case of short-term treatments with very high, nonphysiological concentrations (0.1–1 M range). However, as most non-essential metal ions are toxic to plants from μM concentrations, the influence of such low concentrations on the osmotic potential of the plants is probably negligible, but other, indirect effects on the water status of the plants are probable to occur (reviewed by Poschenrieder and Barceló 2006).

#### 26.3.2.3.4 *Deficiency of Essential Heavy Metals Induced by the Excess of Another Heavy Metal*

It is well documented that indirect injury mechanisms caused by nonessential metals can be based on metal-induced deficiency of Fe, Mn, or other essential micro- or macronutrients. This process might be related to excess metal-induced changes in transpiration, root development, and as a



consequence to decreased water and nutrient uptake of plants, or to substitution or replacement of essential metals in metalloproteins (physiological essential-metal deficiency) or to competitive interactions with nutrient uptake and transport components.

According to the Irving–Williams series, the different metal ions can bind to organic ligands in a metal-binding site of a metalloprotein, metal chaperone or metal transporter with different affinities ( $\text{Zn}^{2+} < \text{Cu}^+ > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+}$ ). At the same time, the binding affinity for a metal ion is also determined by other secondary factors such as the size of metal-binding-site cavity in a protein, the geometry of ligand atoms, and other characteristics, but normally each metal ion can be replaced by other metal ions downstream in the Irving–Williams series (reviewed by Yruela 2009). The potential for Zn, and especially Cu, to displace other metals is relatively high. This replacement is not only true for metal-containing proteins, important for plant functioning, for metal-containing molecules such as Chls (e.g., see Table 26.2), but also for all metal transporters and ion channels within the cells. Similarly, toxic metals may also substitute these ions. This way, it is quite evident that excess metals may induce nutrient deficiency. However, these interactions depend also on the treatment (i.e., if roots or leaves are treated), the form of the metal added, the plant material, and the experimental conditions (concentration of ions, pH, presence of chelators); therefore the results in the literature are difficult to compare (reviewed by Fodor 2002). Different chelators can have a strong influence on uptake, transportation, and/or on apoplasmic or non-apoplasmic accumulation of different interacting metals in the nutrient solution (e.g., Fodor et al. 2005).

One of the best known examples for nonessential metal-induced nutrient deficiency is the chlorosis caused by Cd, which has been shown to be related to Fe-deficiency, rather than to the direct inhibitory effect of Cd (e.g., Sárvári 2005, Fodor 2006, Pál et al. 2006). In plants grown on Fe-deficient nutrient solution, relatively more Cd was translocated into the shoot, and both PSs showed higher sensitivity to Cd (reviewed by Siedlecka and Krupa 1999). On the other hand, elevated Fe supply applied together or after Cd treatment could prevent most Cd effects (Siedlecka and Krupa 1996, Solti et al. 2008, reviewed by Siedlecka and Krupa 1999). Cd may also have indirect effects on PSI and influences the electron transport chain, ferredoxin-dependent  $\text{NADP}^+$  photoreduction, and Chl biosynthesis, by causing Fe-deficiency (reviewed by Siedlecka and Krupa 1999, Pál et al. 2006). Cd also lowers the Chl a/b ratio (Sárvári et al. 1999) due to stronger reduction of PSI than LHCII (Szegi et al. 2007). Cd reduced the amount of Chl-containing complexes in the order of  $\text{PSI} > \text{LHCII} > \text{PSII core}$  (Sárvári et al. 1999) similar to that observed in Fe-deficient plants. The indirect nature of the Cd-induced inhibition of the light phase of photosynthesis and of the different Chl–protein complexes is confirmed by the fact that in Cd-treated plants exhibiting all symptoms of Cd toxicity, the photosynthetic activity could be restored at least partially with the addition of Fe (Solti et al. 2008). Chl fluorescence imaging has shown that the recovery of the photosynthetic activity started from the parts adjacent to the veins and gradually extended to the interveinal parts (Solti et al. 2008) indicating clearly that Cd interfered with Fe uptake and/or transportation in the plants. Therefore Fe-deficiency can be considered as a key factor in Cd-induced inhibition of photosynthesis.

The molecular basis of Cd-induced Fe-deficiency in some plants is that phytosiderophores and other complexing agents excreted by plants can also chelate other metals including Cd and Pb (Strategy II plants; reviewed by Kochian 1995, Hill et al. 2002, Fodor 2006). In Strategy I plants (dicots and nongraminaceous monocots), metallic pollutants interfere with root ferric-chelate reduction (Cd, Pb, Cu, Ni, Mo, Zn) (reviewed by Fodor 2006). However, in some cases, there is no clear correlation between the Fe content of the leaves of metal-treated plants and the decrease in various physiological parameters, suggesting that the total Fe content in the tissues is not necessarily the same one as the active Fe pool (Sárvári et al. 1999, reviewed by Fodor 2002). Cd treatment also induced decreased levels of micronutrients (Cu, Zn, Fe, Mn, Mo, Ni) and macronutrients (Ca, K, Mg, N, P, S) in leaves and roots (reviewed by Fodor 2002, Pál et al. 2006, Chen et al. 2009, Hasan et al. 2009). This process might be related to the fact that Cd was shown to decrease water uptake and thereby the amount of all transported nutrients (cucumber roots: Varga et al. 1999). The Cd-induced Ca-deficiency in pea plants caused the downregulation of Cu/Zn-SOD, leading to the

overproduction of the ROS hydrogen peroxide and superoxide, as monitored *in vivo* by confocal laser microscopy (Rodríguez-Serrano et al. 2009). The production of these ROS was mainly associated with vascular tissue, epidermis, and mesophyll cells, and the production of superoxide radicals could be prevented by exogenous Ca (Rodríguez-Serrano et al. 2009). The toxic effects of Cd were reduced in the presence of elevated amounts of Ca in runner bean plants (Skórzyńska-Polit et al. 1998) and in transgenic tobacco plants transformed with *TaLCT1* (Antosiewicz and Hennig 2004). Addition of Ca to lettuce plants increased tolerance and accumulation of Cd, while La decreased Cd accumulation (reviewed by He et al. 2005). Interestingly, other authors found no effect of Cd on Ca accumulation in rice (Cui et al. 2008) or found positive effect on Ca accumulation in sugar beet (Larbi et al. 2002). Some experiments with lettuce have shown that increasing Cd concentrations increased the Mn uptake (Ramos et al. 2002), while in cucumber (Sárvári et al. 1999) and tomato (Baszyński et al. 1980) the opposite tendency was found. Addition of Mn to the nutrient solution of hydroponically grown Cd-treated tomatoes restored the photosynthetic pigment content and the photosynthetic activity of the seedlings and induced grana formation (Baszyński et al. 1980).

Similarly to Cd, Pb was also shown to cause Fe-deficiency in plants (Wallace et al. 1992, Varga et al. 2002, Sinha et al. 2006) and chlorosis (Sinha et al. 2006, reviewed by Fodor 2002). Pb had an inhibitory effect on Fe-chelate reductase activity in sugar beet (Larbi et al. 2002). In this study, Pb had only a minor effect on other nutrient concentrations. Pb contamination leads to a drastic reduction of Ca accumulation in cucumber roots and a slight increment in the transport of the essential nutrients, especially Mn (Varga et al. 1999, Cseh et al. 2000). In different experiments, Pb decreased Ca, Mn, K, Zn (reviewed by Fodor 2002), and Ni (reviewed by Chen et al. 2009) contents of the plants. In cabbage, Pb decreased the Mn and Cu contents (Sinha et al. 2006).  $\text{Cr}^{3+}$  induces Fe-deficiency in cabbage (Pandey and Sharma 2003). Interestingly, in  $\text{Cr}^{6+}$ -treated bean seedlings, higher Cr concentrations, decreased Chl content, and drastic changes in chloroplast structure were observed, while the plastid ultrastructure and Chl content of primary leaves that accumulated less Cr was almost not affected (Vázquez et al. 1987). Exposure of spruce plants to both organic and inorganic Hg resulted in a loss of K, Mg, and Mn and accumulation of Fe, indicating that essential-nutrient deficiency may also contribute to the toxic physiological effects observed under excess Hg (for a review see Boening 2000).

### 26.3.3 SOME UNUSUAL PHENOMENA ASSOCIATED WITH HEAVY-METAL STRESS

In the literature related to HM stress, there are some data that are not often discussed in metal-stress related reviews. In some cases, the excess of one metal has a positive effect on the uptake of another one. On the other hand, low concentrations of HMs sometimes had a positive effect on plant metabolism. In this section, we briefly summarize these phenomena.

#### 26.3.3.1 When the Excess of a Metal Alleviates the Stress Caused by Another Metal

As stated and illustrated above (Table 26.1), the inhibitory effect of excess metals on plant metabolism are very often related to the fact that they compete for uptake and transportation and induce deficiency in essential nutrients. This is also true for unbalanced concentrations of essential HMs present in the soil, but it is more evident for toxic elements. When the amount of the toxic metal is low, its uptake into the cells or organs is probably rather weak because its chance to be taken up and to be transported is relatively low. The situation may dramatically change when the amount of toxic metal(s) increases in the environment. Additionally, changes in the concentration may also affect the amount of metals passively transported. Therefore, it is not surprising that in several of these cases, the toxic effects and symptoms induced by the metal in excess can be alleviated by the addition of the essential metal with which it is in competition (reviewed by Poirier et al. 2008). This way, the excess metal-induced deficiency, and the disturbed essential metal homeostasis can be recovered. In these cases, the presence of a beneficial element plays an antidote role against the non-beneficial element, even when it is given in excess.

Several experiments have shown that the Fe nutrition status of plants may significantly modify nonessential metal uptake (reviewed by Fodor 2006). Experiments on the interaction of Fe supply with Cd uptake revealed that an overdose of Fe decreases Cd accumulation in chloroplasts of primary bean leaves treated with Cd via the nutrient solution (Siedlecka and Krupa 1996). Similarly, the addition of excess Cu decreased the Cd uptake of rice plants (Cui et al. 2008). A moderate excess of Fe results in increased growth and photosynthetic pigment content and more efficient light phase of photosynthesis in Cd-treated plants as a result of the recovery from Cd-induced physiological Fe-deficiency (Solti et al. 2008, for reviews, see Siedlecka and Krupa 1999, Fodor 2006).

Cd-induced decreased photosynthetic rate, carotenoid and Chl contents, as well as disturbed plastid ultrastructure could be partially recovered by the supplementation of Mn in the growth media; at the same time the Cd content of the different plant organs (leaves, stems, and roots) decreased (Baszyński et al. 1980).

Surprisingly, there are also results indicating the positive effect of toxic or nonessential HMs on the uptake of essential metals; however, the exact molecular background of these observations is not completely clear. Some experiments with lettuce have shown that increasing Cd concentrations increased the Mn uptake (Ramos et al. 2002). Pb contamination of cucumber leads to a slight increment in the transport of the essential nutrients, especially Mn (Varga et al. 1999, Cseh et al. 2000). Increasing Pb concentrations increased the Zn content in cabbage leaves (Sinha et al. 2006). Low concentrations of  $\text{Cr}^{3+}$  restored the chloroplast ultrastructure in Fe-deficient common bean plants (Poschenrieder et al. 1991).

Silicon is known to effectively mitigate various abiotic stresses such as Cd, Mn, and Al pollution, and also salinity, drought, chilling, and freezing. However, mechanisms of Si-mediated alleviation of abiotic stresses remain poorly understood. The key mechanisms of Si-mediated alleviation of abiotic stresses in higher plants include (1) stimulation of antioxidant systems in plants, (2) complexation or coprecipitation of toxic metal ions with Si, (3) immobilization of toxic metal ions in growth media, (4) uptake processes, and (5) compartmentation of metal ions within plants (reviewed by Liang et al. 2007). In rice, the addition of Si to nutrient solutions had a positive effect on plant growth and could decrease the Cd accumulation in the shoots and had therefore an enhancing effect on shoot and root biomass under moderate Cd stress (Zhang et al. 2008).

### **26.3.3.2 When Nonessential Metals Added at Low Concentrations Have a Stimulating Effect**

The toxic and damaging effects of metals are usually observed when the stressors are applied at relatively high concentrations ( $10^{-5}$ – $10^{-3}$  M). Nevertheless, these harmful compounds used at low concentrations (defined as “low-concentration stressors,”  $10^{-8}$ – $10^{-6}$  M) may have a beneficial effect on plants. However, the effect depends on the developmental stage of the plant at the beginning of the treatment. The low-concentration stressors stimulate biosynthetic processes and growth in seedlings, and delay aging in detached leaves, which is reflected also by an increased metabolism (Nyitrai et al. 2007, 2009, Kovács et al. 2009). The stimulating effect is nonspecific, i.e., independent of the agent used (Nyitrai et al. 2003, 2004).

The acceleration of plant growth is one of the stimulatory effects of nonessential metals observed in these studies. Short-term Cd treatments of maize roots result in small growth stimulation (Wójcik and Tukendorf 1999). Similarly, long-term treatments with Pb, Ni, and Ti induce a significant increase in root length, dry and fresh weights, and shoot growth in maize and bean (Nyitrai et al. 2003).

Low-concentration stressors often stimulate the metabolic activity, i.e., respiration, Chl biosynthesis, and photosynthetic activity in seedlings (Prasad et al. 2001, Nyitrai et al. 2004, Nyitrai et al. 2007). At the germination stage, Cd and Pb significantly increase the levels of Chls and carotenoids until the fourth day of development (Shaw and Rout 1998). Similarly, at relatively low concentrations, Pb has a slight stimulatory effect on the Chl content of cucumber leaves (Sárvári et al. 1999). Cd treatment of young maize seedlings increases the leaf Chl (Dratzkiewicz et al. 2003,

Drazkiewicz and Baszyński 2005) and carotenoid (Drazkiewicz and Baszyński 2005) content up to 100  $\mu\text{M}$ , but causes chlorosis when applied above this concentration. Low concentrations of  $\text{C}^{3+}$  enhance the growth of bean plants and reduce the chlorosis in young leaves of Fe-deficient plants by increasing the concentration of Chls and carotenoids (Bonet et al. 1991, Poschenrieder et al. 1991).

One of the most comprehensive surveys in this field is that of Nyitrai et al. (2003). In this study, low concentrations of different metals (Cd:  $5 \times 10^{-8}$  and  $10^{-7}$  M, Pb and Ni:  $10^{-7}$  and  $10^{-6}$  M, Ti:  $10^{-6}$  and  $10^{-5}$  M) had a stimulating effect on Chl biosynthesis and photosynthetic activity of bean and maize. Treatments applied either in the nutrient solutions or by spraying the leaves were both effective. However, the extent of the stimulating effect depended on the species, the time course of treatment, the position of leaf, and the agent (Nyitrai et al. 2003). The stimulation of the photosynthetic activity was observed at different intervals during all treatments (Cd, Pb, Ni, Ti), while Chl a/b ratios of leaves or chloroplasts did not change considerably (Nyitrai et al. 2003). Low-concentration stressors increased the amount of PSI and LHCII. Electron microscopy of either maize or bean leaves did not show significant differences in the chloroplast lamellar systems between control and treated plants. The authors concluded that these low-concentration stressors generate nonspecific alarm reactions in plants, which may involve changes of the hormonal (e.g., cytokinin) balance (Nyitrai et al. 2003, 2004). In a later study, the authors have demonstrated that in barley seedlings treated with low concentrations of Cd ( $5 \times 10^{-8}$  M), the amount of cytokinins increases in the roots, and it is transported to the leaves where it also causes stimulation (Kovács et al. 2009). The phosphatidylinositol-4,5-bisphosphate-inositol-1,4,5-triphosphate/diacylglycerol and the mitogen-activated protein kinase signaling pathways, and not the stressor itself, were found to be responsible for the primary stimulation of cytokinin synthesis and/or activation in the roots (Kovács et al. 2009). Cd treatment at this low concentration did not induce any oxidative stress either in the roots or in the leaves, where Cd did not even accumulate to detectable amounts.

In model systems (i.e., the detached old leaves of different plants), the beneficial effect of the moderate and weak stress that induces nonspecific alarm responses in plants could be studied and was shown to have a rejuvenating effect on plastids and their metabolism (Nyitrai et al. 2007, 2009). The physiological parameters of detached leaves are stimulated in a similar way to those observed in the case of seedlings (Nyitrai et al. 2003, Kovács et al. 2009). At the same time, the stressors stimulate starch accumulation in the chloroplasts, and a decrease of the large plastoglobuli typical for plastid senescence (Nyitrai et al. 2004, 2007). Under Pb ( $10^{-7}$  M) and Ti ( $10^{-6}$  M) treatment of detached, non-rooting barley leaves, the level of active cytokinins is not affected, indicating the direct effect of the stressors in this experimental system (Nyitrai et al. 2007). In both model systems (detached bean and barley leaves) the phosphatidylinositol-4,5-bisphosphate-inositol-1,4,5-triphosphate/diacylglycerol signaling pathway is involved in the anti-senescence effect (Nyitrai et al. 2007, 2009). These interesting data provide further evidences for the complexity of interactions of plastids and metals.

## 26.4 CONCLUSION

In case of metal pollution in the environment, an unbalanced metal content is often observed in plastids: potentially toxic HMs are present and essential elements are missing. Thanks to various strategies, the cells can cope with a moderate unbalance, but in case of strong unbalance, too many essential biomolecules are not functional any more, photosynthesis is altered and plant productivity is decreased. Although sufficient data are available on HM transporters, it appears that further studies on the mechanism of HM uptake and regulation in plastids are needed. Also, the metal translocation in the whole plant cell, and especially the transporters of the tonoplast that favor the elimination of toxic elements should be further studied. Moreover, the visualization of metal movement and transport at the cellular scale would greatly help. A possibility of preventing the entrance of the nonessential elements in plastids would be to produce mutants with more metal-specific transporters. The mechanisms allowing non-essential metals to have a stimulating effect on plant growth should be also further investigated.

## ABBREVIATIONS

|                          |                                                                     |
|--------------------------|---------------------------------------------------------------------|
| ATP                      | Adenosine triphosphate                                              |
| Chl                      | Chlorophyll                                                         |
| DGDG                     | Digalactosyl-diacylglycerol                                         |
| DNA                      | Deoxyribonucleic acid                                               |
| HM                       | Heavy metal                                                         |
| HMA                      | HM ATPase                                                           |
| LHC                      | Light-harvesting complex                                            |
| MGDG                     | Monogalactosyl-diacylglycerol                                       |
| NADP <sup>+</sup> /NADPH | Nicotinamide adenine dinucleotide phosphate (oxidized/reduced form) |
| OEC                      | Oxygen-evolving complex                                             |
| PS                       | Photosystem                                                         |
| RNA                      | Ribonucleic acid                                                    |
| ROS                      | Reactive oxygen species                                             |
| RUBISCO                  | Ribulose biphosphate carboxylase oxygenase                          |
| SOD                      | Superoxide dismutase                                                |

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# 27 Plant Responses to Cadmium and Mercury Stress

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## 27.1 INTRODUCTION

In view of the increasing anthropogenic impact on the environment, the problem of emissions of heavy metals (HMs) gets more and more attention. Study on interaction effects between HM and biomolecules is of great theoretical and practical importance as it concerns economical and social life spheres, such as medicine, eco-toxicology, agriculture, recycling of wastes, etc. Despite the numerous reviews and reports on HM in literature, the study of phytotoxicity mechanisms is still incomplete (Masarovičová et al. 1999, Prasad and Hagemeyer 1999, Titov et al. 2007).

HMs are a group of metals with relative atomic weight over 50 or density higher than  $5 \text{ g cm}^{-3}$ . Pollutants mostly contain arsenic, cadmium, cobalt, chromium, copper, mercury, lead, nickel, and zinc. Several HMs such as iron, manganese, zinc, copper, nickel, and molybdenum are required by plants as micronutrients. Their concentrations in plant dry matter vary from 100 to 0.1 ppm (Taiz and Zeiger 2002). Mn is required for activity of some dehydrogenases, decarboxilases, oxidases, and peroxidases and is involved with other cation-activated enzymes and photosynthetic  $\text{O}_2$  evolution.



Iron is a constituent of cytochromes and nonheme iron proteins in photosynthesis,  $N_2$  fixation, and respiration. Zinc and Cu are components of many enzymes involved in redox reactions as well. Molybdenum is a constituent of nitrogenase, nitrate reductase, and xanthine dehydrogenases. Nickel has been recently recognized as an important plant micronutrient as a component of urease.

Other HMs such as cadmium, lead, mercury, arsenic, etc., are not essential for plants and are highly phytotoxic. HM concentration in soil is agreed to be toxic when it inhibits plant growth and development and decreases plant productivity by 10%–20% (Zyrin and Sadovnikova 1985). However, the identification of toxic levels of HM for plants is a very complicated problem. Knowledge of environmental conditions, HM forms and their stability in the environment, plant properties, etc., are of great importance for the assessment of HM toxicity.

In this chapter, we have paid special attention to the phytotoxicity of two HMs—cadmium and mercury. By the resolution of the Environmental Protection Agency, these metals belong to the group of important environmental pollutants. They represent maximum potential danger for animals and people as they have a high cumulative effect and high rate of technogenic accumulation in the environment (Maistrenko et al. 1996, Chernykh and Ovcharenko 2002). According to the phytotoxicity classification, mercury is a very phytotoxic element as it inhibits the life activity of test organisms at concentrations in medium near ( $1 \text{ mg L}^{-1}$ ); cadmium is a moderately toxic element as it inhibits the life activity of test organisms at concentrations  $1\text{--}100 \text{ mg L}^{-1}$  (Alexeev 1987). Cadmium and mercury are mobile in plants, and are accumulated in all plant organs, and seeds as well (Kabata-Pendias and Pendias 1984). Plants can volatilize mercury through leaves in the form of dimethylmercury, but dimethylmercury is synthesized 6000 times slower than methylmercury, which is very capable of penetrating through biological membranes (Gay et al. 1978).

## 27.2 CHARACTERISTICS OF CADMIUM AND MERCURY

Cadmium and mercury are chemical elements belonging to one triad group (Zn, Cd, Hg) (12) of the periodic table (IUPAC style). The standard atomic weight of Cd is  $112.411 \text{ g mol}^{-1}$  (atomic number 48), and that of Hg is  $200.59 \text{ g mol}^{-1}$  (atomic number 80).

### 27.2.1 CADMIUM

Cadmium is the soft, bluish-white bivalent metal, whose melting point is  $321^\circ\text{C}$  and whose density is  $8.65 \text{ g cm}^{-3}$ . It is similar in many respects to zinc but forms more complex compounds. The most common oxidation state of the metal is +2. It has high affinity to sulfur, to SH groups in particular, which is responsible for a high accumulation of cadmium in living cells and its compounds' toxicity.

Cadmium is a relatively abundant element. The weight fraction of cadmium in the lithosphere varies around  $n \cdot 10^{-5}\%$ . Cadmium and zinc usually compose carbonate and sulfide ores, argillaceous sediments, and in shales.

The role of cadmium in biology has been recently discovered (Lane et al. 2005). Cadmium-dependent carbonic anhydrase, which is involved in supplying inorganic carbon for photosynthesis, has been found in the marine diatom *Thalassiosira weissflogii*. It is known that the diatoms live in environments with very low zinc concentration, and cadmium can substitute Zn in an active site of the enzyme in vivo.

World cadmium production makes about  $1.4 \cdot 10^4 \text{ t year}^{-1}$ . Cadmium is produced as by-product when other metals are refined. Cadmium was for a long time used as pigment and for corrosion-resistant plating on steel. Cadmium compounds were used to stabilize plastics (Scoullou et al. 2001).

### 27.2.2 MERCURY

Mercury is one of the six chemical elements that are liquid at or near room temperature and pressure, its melting point being  $-38.83^\circ\text{C}$  and density (liquid) being  $13.534 \text{ g cm}^{-3}$ . Mercury ions

can form complex compounds with coordination numbers from 2 to 8. Mercury dissolves to form amalgams with gold, zinc, and many other metals.

The weight fraction of mercury in the lithosphere equals  $n \cdot 10^{-6}\%$ – $10^{-5}\%$ . In the environment, mercury occurs as  $\text{Hg}^0$ ,  $\text{Hg}^{1+}$ , and  $\text{Hg}^{2+}$ . Native mercury, as inclusions in mountain rocks, is rare in occurrence. In nature, it exists in the form of bright red  $\text{HgS}$ , or cinnabar. This mineral is used for making red color (Trakhtenberg and Korshun 1990, Scoullos et al. 2001).

World mercury production makes  $8.4 \cdot 10^3 \text{ t year}^{-1}$ . Mercury is used for producing thermometers, barometers, and manometers. Mercury vapor fills mercury-quartz and fluorescent lamps. Mercury contacts serve as position sensors. Metal mercury is applied for production of many important alloys, etc. (Trakhtenberg and Korshun 1990, Scoullos et al. 2001).

### 27.2.3 TOXICOLOGY OF CADMIUM AND MERCURY

Owing to similar electronic structure, cadmium and mercury are thought to have similar effects on the biochemical systems of animals and humans. Both metals provoke calcium release from nephrocytes and hepatocytes and so cause the membrane potential decrease (Malis and Bonventre 1988). They are highly nephrotoxic as they accumulate in parenchymatous organs, in renal cortex especially, and remain in the organism for a long time (Ambrosi et al. 1991). Affected by cadmium and mercury, lipid peroxidation increases in human erythrocytes (Malis and Bonventre 1988).

All cadmium and mercury compounds, as well as vapor, are toxic. Blood-absorbed HMs affect the nervous system, liver, and kidneys, and inhibit phosphorus-calcium exchange. When inhaled, mercury vapor affects respiratory tracts (mercury enters human organism usually as odorless vapor) (Chernykh and Ovcharenko 2002). In 1956, the so-called Minamata disease was reported that first appeared in South Japan because of methylmercury poisoning. This disease has symptoms as deformation of extremities, breathing difficulty, paralysis, and convulsions (Timothy 2001). Chronic cadmium poisoning causes anemia, decalcification, and bones' destruction (Itai-itai disease) (Nogawa and Kido 1996).

## 27.3 HEAVY METAL ENVIRONMENTAL POLLUTION

### 27.3.1 HEAVY METAL RELEASE INTO ENVIRONMENT

There are natural and technogenic sources of HM release into the environment (Kabata-Pendias and Pendias 1984, Alexeev 1987). Among natural sources, there are mountain rocks and minerals' weathering, volcanic activity, and soil erosion. The other much stronger source of HM release is produced by anthropogenic factors (metal-working industries, power station, mining industry, cement factories, urban traffic, by-product of fertilizers, etc.). In areas with high anthropogenic pressure, HMs are 100 times more often involved in biochemical cycle as compared with natural HMs (Pacyna et al. 1984). World anthropogenic emissions of cadmium and mercury are equal to about  $30 \cdot 10^3$  (Sanità di Toppi and Gabrielli 1999) and  $8 \cdot 10^3 \text{ t year}^{-1}$  (Trakhtenberg and Korshun 1990), respectively.

Land ecosystems are most technogenically impacted. Natural HM contents in soils have a great diapason of variation, depending on HM concentration in native rocks, on relief, and climate. For example, mercury concentration in surface soil layer varies within 0.01–0.9, cadmium 0.01–2.5, nickel 2–300, and lead 2–50  $\text{mg kg}^{-1}$  (Kabata-Pendias and Pendias 1984, Chernykh and Ovcharenko 2002). HM contents in high-polluted areas exceed the above-cited values by tens of hundred times.

### 27.3.2 ASSESSMENT OF HEAVY METAL ENVIRONMENTAL POLLUTION

Under the steady increasing anthropogenic pressure on nature, it is important to assess the ecological situation with HM distribution in the environment. Assessment of soil pollution (for air and

snow as well) is based on pollution comparison of city soils with background soils (in areas with low anthropogenic pressure). For this, concentration coefficient ( $C_c$  or  $C_{cl}$ ) as ratio between HM content in city and background soils is applied (Chernykh and Ovcharenko 2002). The  $C_c$  explains the pollution intensity but does not take into consideration a danger of the pollution for living organisms. Therefore, the value of maximum concentration limit (MCL) experimentally determined for every HM is applied for ecological and sanitary-hygienic assessment of soil pollution. The MCL of a pollutant means the maximum amount of the pollutant which does not have negative direct or indirect effect on human health, future generation, and sanitary life conditions (Chernykh and Ovcharenko 2002).

The MCL values are not the same in different countries and are regularly revised. For example, MCL for cadmium and its organic compounds in drinking water in Russia is 0.001, in air 0.05 mg dm<sup>-3</sup>, and in soil 2 mg kg<sup>-1</sup>. MCL for mercury in drinking water is 0.0005, in air 0.005 mg dm<sup>-3</sup>, and in soil 2.1 mg kg<sup>-1</sup> (Chernykh and Ovcharenko 2002).

At present, the probabilistic approach proposed by the U.S. EPA (Environmental Protection Agency) in the early 1980s is widely used. This concept ("risk assessment") considers the combined effect of pollutants and additional parameters (sex, age, genetic features of studied population).

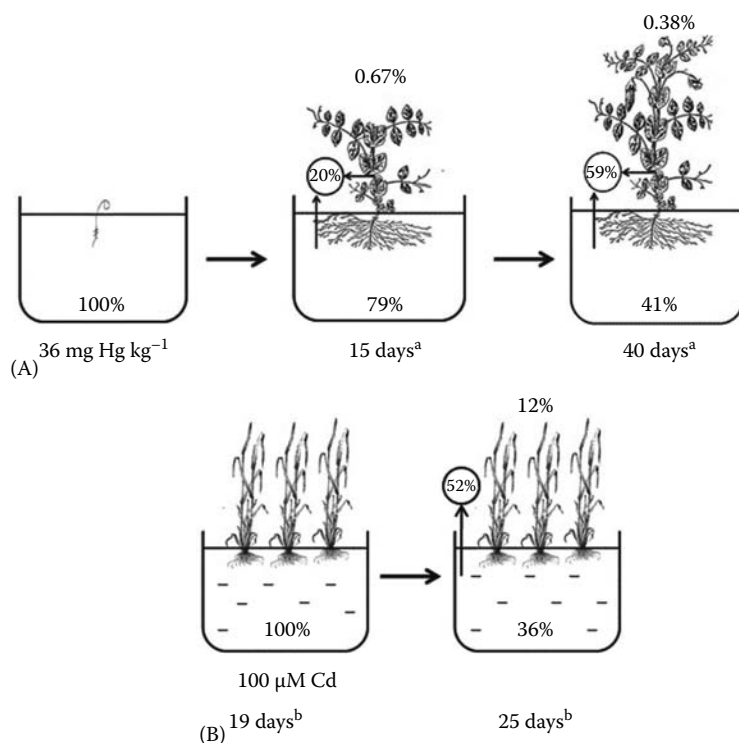
Based on the environmental quality monitoring analysis performed in areas of the industrial Kirov's district (Kirov is a high industrially developed city), HM contents in soils and wild plants often exceed their  $C_c$  and MCL values. Mercury soil content was under MCL but 10–20 times as higher than mercury  $C_c$  (0.08), especially near the chemicals industrial complex. The content of mercury, cadmium, and other HM exceeded MCL values near the polymers plant (Skugoreva et al. 2008). It is well known that the main polluters and source of such HMs such as cadmium, lead, and copper in the Komi Republic (the Russian Arctic and subarctic) are discharge of coal mining and reprocessing, oil and gas industries (Walker et al. 2009).

### 27.3.3 HEAVY METAL MIGRATION PROCESSES

Knowledge on HM migration processes and on availability of different HM compounds in the environment is required for the HM toxicity assessment. Labile HM forms (water-soluble, exchangeable, acid-soluble HM compounds with organic and nonorganic ligands) are most dangerous because of their high biochemical activity and intensive accumulation in the environment and living organisms. According to the biogeochemical properties, cadmium, mercury, and lead simultaneously have high rates of mobility, aerosol enrichment, accumulation intensity, solubility, and bioconcentration capability (Roeva et al. 1996).

Migration processes of HM largely depend on soil properties such as humus content, dispersion, and pH. Within the capability, soils absorb HM anions, and HMs acquire inactive state. Soil colloids have negative charge. They have hydroxyl groups and electron pairs of oxygen, as well as carboxylic and phenol groups of organic substances. Metal anions are attracted by soil colloids. The uptake of metals by plant roots increases with increasing pH in soil solution, which is due to competition between hydrogen ions (they have a higher affinity to negative-charged soil colloids) and metal ions at the bonding sites in colloids (Greger 1999). Soil organic matter lowers the mobility of metal ions by bonding metals to fulvic and humic acids. Clay and/or humus-enriched soils with high pH are generally known for bonding more metal ions and holding them for longer time, compared to other soil types. Cadmium in acid solution is more mobile than zinc (Chernykh and Ovcharenko 2002), and it can be highly toxic in acid soils, for example, podzolic soils in the North (the Komi Republic) (Zaboeva et al. 2002).

It has been demonstrated that HM uptake rate by plants from hydroponic culture was higher than HM uptake from soil. Cd content in the hydroponic culture, where barley plants treated by 100 μM Cd were growing, in 6 days was 36% of initial concentration (Figure 27.1). Twelve percent of metal was taken up by plants, and the rest (52%) evaporated. On plots outside, where plants (pea, oat) were growing on fine-textured loamy arable podzolic soil (pH 5.7), soil Cd content did not essentially



**FIGURE 27.1** Dynamics of mercury in a soil-plant system (A) and cadmium in a nutrient solution-plant system (B) (with *Pisum arvense* L., cv. Nadezhda (A), and *Hordeum distichum* L., cv. Novichok (B) as examples). <sup>a</sup>Days after seedlings appearance, <sup>b</sup>days after seeding. Mercury was added to a pot-filled ground in the form of  $\text{Hg}(\text{NO}_3)_2$ . (From Skugoreva, S.G. and Golovko, T.K., *Agrokimiya (Agrochemistry)*, 2, 66, 2007b, in Russian.)

change, and annual Cd uptake by plants was less than 1% (Elkina 2007). Plants of *Pisum arvense* L. (cv. Nadezhda) were grown in soil substrate with initial mercury content in form of  $\text{Hg}(\text{NO}_3)_2$  equal to  $36 \text{ mg Hg kg}^{-1}$ . In 40 days of the experiment, the content of mercury in soil was 40% of the initial value. Only 0.4% of mercury was taken up by plants (Figure 27.1) (Skugoreva and Golovko 2007a). It is important that the soil and plants can volatilize Hg in form of dimethylmercury in significant quantities (Gay et al. 1978). Up to 6% of total mercury content per day can evaporate if soluble mercury salts were added to soil (Rogers and McFarlane 1979).

Potentially human-dangerous food chains usually begin with polluted crop production. According to the Sanitary Rules and Norms in Russia, the MCL for Cd in plant green mass and grain is 0.03 and  $0.05 \text{ mg kg}^{-1}$  fresh weight, respectively. The MCL for mercury is 0.02 in vegetables and  $0.03 \text{ mg kg}^{-1}$  fresh weight in grain (Chernykh and Ovcharenko 2002). It was shown that plants accumulate human-dangerous concentration of mercury even under low Hg soil pollution (less than  $18 \text{ mg Hg kg}^{-1}$  soil).

## 27.4 HEAVY METAL PHYTOTOXICITY

Excess HM, even of the necessary microelements, is toxic for plants. Knowledge of the mechanisms of metal toxicity in plants is still not well known. It is known that HM can bind to functionally important domains of biomolecules and thereby inactivate them. The result is the substitution of a micronutrient by toxic HM, deactivation and depolymerization of a macromolecule, improper protein synthesis and its replication, transcription, translation of genome, and production of the toxic substances underlies the HM toxicity (Bingham et al. 1986). Cadmium and mercury can cause cell death in plants by inactivating enzymes and structural proteins by acting mostly on sulphhydryl

groups. About 100 enzymes are known to bind free metals through sulphhydryl groups and become inactive, which is the cause of disturbances in cell metabolism.

### 27.4.1 GROWTH

Growth changes are often the first and most obvious reactions of plants under stress (Hagemeyer 1999). As a rule at high HM treatments, plant growth is significantly reduced due to inhibition of cell division and decrease of cell wall elasticity through accelerating the lignifications processes (Sanità di Toppi and Gabrielli 1999, Seregin and Ivanov 2001). Cd and other HMs induced mitotic disturbance, which resulted in increase in amount of fissionable cells in metaphase state (Seregin and Ivanov 2001). Different chromosomal aberrations and inhibiting spindle system in *Allium cepa* L root tips treated by mercuric fungicides were noted (Nandi, 1985). In our experiments, high cadmium concentration affected strongly growth processes: relative growth rate of barley plants decreased by 2–3 times (Table 27.1). Cool temperature regime enhanced the metal impact on plant growth evidently due to suppression of Cd immobilization and detoxification (Garmash and Golovko 2009). Lettuce plants were more sensitive to mercury impact (Table 27.2). Plants grown in soil substrate containing 36 mg Hg kg<sup>-1</sup> decreased biomass twice compared to those under the control level; higher mercury concentration induced plant death. Up to 70% of growth reduction was induced by the high mercury concentration in garden radish and garden-cress plants.

### 27.4.2 PHOTOSYNTHESIS

HMs are reported to have adverse effect on photosynthetic apparatus (Titov et al. 2007, Greger and Ögren 1991, Krupa and Barzyński 1995, Prasad and Strzałka 1999, Mobin and Khan 2007). HMs disturb chlorophylls' biosynthesis and chloroplasts' structure, provoke the photosystems' perturbation, inhibit the photosynthetic carbon reduction cycle enzymes, and interfere in stomatal and gas functions. The substitution of the central Mg in chlorophyll by HM (Hg, Cd, Cu, Ni, Zn, Pb) in vivo is an important type of damage in metal-stressed plants (Prasad and Strzałka 1999, Patra and Sharma 2000). According to our data (Figure 27.2), chlorophylls content in leaves under Cd and Hg impact decreases from 15% to 50%, depending on the external metal concentration due to likely inhibiting chlorophyll biosynthesis enzymes, in particular 5-aminolevulinic acid dehydrogenase and protochlorophyllide reductase (Stobart et al. 1985, Baryla et al. 2001, Mysliwa-Kurdziel and Strzałka 2004). High Cd concentration inhibited net photosynthesis (Figure 27.3). Carotenoids as pigments with high antioxidative capability were less affected by HM, which was observed in other studies (Krupa 1988, Khudsar et al. 2001, Lunačkova et al. 2003).

### 27.4.3 RESPIRATION

Mitochondria are thought to be more resistant to HM effects than chloroplasts. Moderate HM concentrations do not affect or even enhance respiration, whereas high concentrations reduce the intensity of this process due to enzyme inactivation (van Assche and Glijsters 1990, Chugh and Sawhney 1999) and disturbances in electron-transport chain (ETC) (Miller et al. 1973, Kessler and Brand 1995). The effects of HM on individual enzymes of the citrate cycle are considered in detail and summarized (Lösch and Köhl 1999). In particular, HMs affect strongly the succinate dehydrogenase complex, malate dehydrogenase, and isocitrate dehydrogenase supplying reduced coenzymes (NADH, FADH<sub>2</sub>) into mitochondrial ETC. There is a little information about the effect of HM on the respiratory pathways ratio in the ETC. It is known that cytochrome oxidase activity is reduced by Cd and Hg (Vallee and Ulmer 1972). Engagement of alternative respiratory pathway (AP) as

TABLE 27.1

**Morphophysiological Parameters and Cadmium Accumulation in the Organs of 25-Day-Old Barley Plants (*Hordeum distichum* L., cv. Novichok) Grown under Two Temperature Regimes (Day/Night) and Different CdSO<sub>4</sub> Concentrations in Nutrient Solution**

| Parameter                                                                              | 21°C/17°C     |                         |                         |                         | 13°C/8°C                |                          |                          |                          |
|----------------------------------------------------------------------------------------|---------------|-------------------------|-------------------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|
|                                                                                        | Control       | Cd Concentration (μM)   |                         |                         | Control                 | Cd Concentration (μM)    |                          |                          |
|                                                                                        |               | 30                      | 60                      | 100                     |                         | 30                       | 60                       | 100                      |
| Plant dry weight (W, g)                                                                | 0.110 ± 0.008 | 0.096 ± 0.008           | 0.090 ± 0.007           | 0.098 ± 0.007           | 0.087 ± 0.006           | 0.058 ± 0.004            | 0.062 ± 0.004            | 0.057 ± 0.003            |
| Relative growth rate<br>( <i>R<sub>G</sub></i> , g g <sup>-1</sup> day <sup>-1</sup> ) | 0.15 ± 0.04   | 0.07 ± 0.03             | 0.05 ± 0.03             | 0.03 ± 0.06             | 0.13 ± 0.05             | 0.02 ± 0.02 <sup>a</sup> | 0.03 ± 0.02 <sup>a</sup> | 0.01 ± 0.04 <sup>a</sup> |
| Malonic dialdehyde, (MDA, nmol g <sup>-1</sup> FW)                                     |               |                         |                         |                         |                         |                          |                          |                          |
| Leaf                                                                                   | 35.7 ± 0.6    | 36.2 ± 1.0              | 32.3 ± 2.9              | 27.5 ± 3.7              | 24.0 ± 5.3              | 33.8 ± 4.1               | 21.6 ± 2.9               | 32.3 ± 1.7               |
| Root                                                                                   | 12.8 ± 1.6    | 24.5 ± 0.5 <sup>a</sup> | 24.1 ± 0.3 <sup>a</sup> | 20.2 ± 0.9 <sup>a</sup> | 25.8 ± 1.1 <sup>b</sup> | 29.6 ± 4.4               | 30.1 ± 2.2               | 40.3 ± 2.4 <sup>a</sup>  |
| Cd content, (mg kg <sup>-1</sup> DW)                                                   |               |                         |                         |                         |                         |                          |                          |                          |
| Shoot                                                                                  | —             | 160 ± 50                | 160 ± 50                | 170 ± 20                | —                       | 120 ± 40                 | 40 ± 50                  | 506 ± 65                 |
| Root                                                                                   | —             | 4,100 ± 140             | 5,600 ± 200             | 15,240 ± 1,500          | —                       | 3,800 ± 130              | 5,050 ± 170              | 10,597 ± 106             |

Source: Garmash, E.V. and Golovko, T.K., *Russ. J. Plant Physiol.*, 56, 343, 2009.

Notes: Significant differences between the control and Cd-treated plants are indicated by <sup>a</sup>(*p* ≤ 0.05), for W, *R<sub>G</sub>* = 30, for MDA, *n* = 6, Cd content, *n* = 3. Significant differences from plants grown at temperature regime 21°C/17°C are indicated by <sup>b</sup>(*p* ≤ 0.05).

**TABLE 27.2**  
**Effect of  $\text{Hg}(\text{NO}_3)_2$  Soil Pollution on Plant Organs**  
**Biomass Accumulation (g DW Plant<sup>-1</sup>)**

|                                                                                               |               | Hg Treatments (mg kg <sup>-1</sup> ) <sup>d</sup> |                            |
|-----------------------------------------------------------------------------------------------|---------------|---------------------------------------------------|----------------------------|
| Species                                                                                       | Control       | 36                                                | 90                         |
| Garden-cress ( <i>Lepidium sativum</i> L.) 30 day-old plants                                  |               |                                                   |                            |
| Root                                                                                          | 0.066 ± 0.011 | 0.028 ± 0.004 <sup>a</sup>                        | 0.018 ± 0.002 <sup>a</sup> |
| Shoot                                                                                         | 0.22 ± 0.03   | 0.22 ± 0.02                                       | 0.18 ± 0.02                |
| Lettuce ( <i>Lactuca sativa</i> L., cv. Moskovskii parnikovyi) 50-day-old plants              |               |                                                   |                            |
| Root                                                                                          | 0.060 ± 0.011 | 0.032 ± 0.008 <sup>a</sup>                        | Plant death                |
| Shoot                                                                                         | 0.85 ± 0.13   | 0.21 ± 0.06 <sup>c</sup>                          |                            |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. 18 days) 40-day-old plants |               |                                                   |                            |
| Root                                                                                          | 0.045 ± 0.012 | 0.034 ± 0.007                                     | 0.014 ± 0.004              |
| Shoot                                                                                         | 0.56 ± 0.14   | 0.40 ± 0.07 <sup>b</sup>                          | 0.29 ± 0.06 <sup>c</sup>   |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. Sofit) 40-day-old plants   |               |                                                   |                            |
| Root                                                                                          | 0.022 ± 0.005 | 0.021 ± 0.004                                     | 0.027 ± 0.004 <sup>a</sup> |
| Shoot                                                                                         | 1.08 ± 0.20   | 0.74 ± 0.19                                       | 0.72 ± 0.14                |

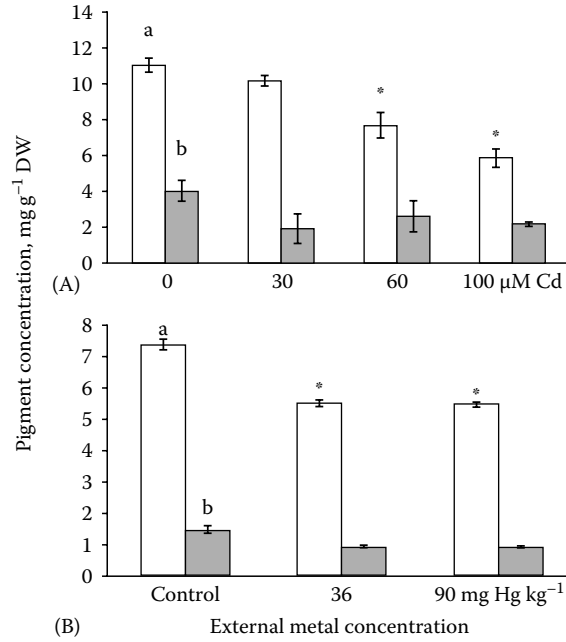
Sources: Skugoreva, S.G. and Golovko, T.K., *Agrokhimia* (Agrochemistry), 5, 85, 2007a, in Russian; Skugoreva, S.G. and Golovko, T.K., *Agrokhimia* (Agrochemistry), 2, 66, 2007b, in Russian.

Notes: Significant differences between the control and Hg-treated plants are indicated by <sup>a</sup> ( $p \leq 0.05$ ), <sup>b</sup> ( $p \leq 0.01$ ), or <sup>c</sup> ( $p \leq 0.001$ ), respectively,  $n = 4$ . <sup>d</sup>  $\text{Hg}(\text{NO}_3)_2$  pollution levels (36 and 90 mg Hg kg<sup>-1</sup> DW) correspond to 10 and 25 of MCLs of Hg in soil, respectively.

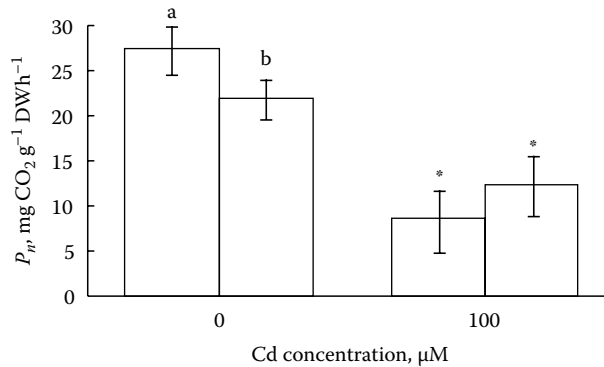
alleviating the formation of reactive oxygen species (ROS) way under Cd effects was observed (Garmash and Golovko 2009). AP activation is believed to be one of the mechanisms for maintenance of root cell homeostasis under cadmium-induced stress.

#### 27.4.4 METAL-INDUCED OXIDATIVE STRESS

HMs stimulate the formation of ROS, either by direct electron transfer involving metal cations or as a consequence of metal-mediated inhibition of metabolic reactions (Dietz et al. 1999). These ROS generated by aerobic metabolism include singlet oxygen ( $^1\text{O}_2$ ), superoxide ions ( $\text{O}_2^-$ ), and peroxides, the most widely distributed being hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Plants in their own life use ROS as signaling molecules, especially in response to various stresses or threats to the plant integrity, as pathogen attacks, or nonoptimal growth conditions (Foyer and Noctor 2003). These molecules can thus act as messengers to trigger protein deactivation or induce the transcription of specific sets of genes in plant cells (Gadjev et al. 2006). On the other hand, plants need to control the levels of these oxidants because of their harmful nature. Therefore, plants have evolved a complex antioxidant defense system (antioxidative enzymes and nonenzymatic antioxidants), which can be regulated according to the environmental conditions. Under non-stress or moderate stress conditions, the antioxidant system is sufficient to ensure redox homeostasis and thus prevent oxidative damage (Dietz et al. 1999). If the antioxidative machinery cannot manage the increased rate of ROS formation, uncontrolled oxidation and radical chain reactions will result in “oxidative blast” (Minibaeva and Gordon 2003).



**FIGURE 27.2** Effects of cadmium (A) and mercury (B) on pigments concentration in plant leaves. a—chlorophylls, b—carotenoids. A—25-day-old barley plants (*Hordeum distichum* L., cv. Novichok) after 6 days of CdSO<sub>4</sub> treatment; B—40-day-old garden radish plants (*Raphanus sativus* var. *radicula*, cv. 18 days). Mercury was added to a soil in the form of Hg(NO<sub>3</sub>)<sub>2</sub>. Significant differences between the control and metal-treated plants are indicated by \* ( $p \leq 0.05$ ),  $n = 10$ . (From Skugoreva, S.G. and Golovko, T.K., *Agrokhimia (Agrochemistry)*, 5, 85, 2007a, in Russian.)



**FIGURE 27.3** Effect of cadmium on net photosynthesis rate ( $P_n$ ) in leaves of 25-day-old barley plants (*Hordeum distichum* L., cv. Novichok) after 6 days of CdSO<sub>4</sub> treatment under two temperature regimes (day/night): 13°C/8°C (a) and 21°C/17°C (b). Significant differences between the control and Cd-treated plants are indicated by \* ( $p \leq 0.05$ ),  $n = 6$ .

ROS attack all important biomolecules, including nucleic acids, proteins, lipids, and amino acids. As a consequence of oxidative damage, the lipid peroxidation, plasmalemma and cytoskeleton destruction, chloroplasts and mitochondria degradation, and inhibition of photosynthesis increase in the presence of toxic metal concentrations (Dietz et al. 1999). The possible result is the cell disorganization and death. In our experiments, the increased accumulation of malondialdehyde (MDA)



**TABLE 27.3**  
**Malonic Dialdehyde Content in the Organs of Some**  
**Plant Species (nmol g<sup>-1</sup> FW) under Different Hg(NO<sub>3</sub>)<sub>2</sub>**  
**Soil Pollution**

| Species                                                                                                                                                                                                                                                                                                                                                                                         | Control      | Hg Treatments (mg kg <sup>-1</sup> ) <sup>d</sup> |                          |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|---------------------------------------------------|--------------------------|
|                                                                                                                                                                                                                                                                                                                                                                                                 |              | 36                                                | 90                       |
| Garden-cress ( <i>Lepidium sativum</i> L.) 30-day-old plants                                                                                                                                                                                                                                                                                                                                    |              |                                                   |                          |
| Leaf                                                                                                                                                                                                                                                                                                                                                                                            | 95.2 ± 3.3   | 101.9 ± 3.3                                       | 103.5 ± 1.7              |
| Root                                                                                                                                                                                                                                                                                                                                                                                            | 40.9 ± 2.8   | 48.8 ± 1.0 <sup>a</sup>                           | 61.2 ± 3.3 <sup>a</sup>  |
| Lettuce ( <i>Lactuca sativa</i> L., cv. Moskovskii parnikovyi) 50-day-old plants                                                                                                                                                                                                                                                                                                                |              |                                                   |                          |
| Leaf                                                                                                                                                                                                                                                                                                                                                                                            | 116.9 ± 8.4  | 145.3 ± 1.7 <sup>b</sup>                          | Plant death              |
| Root                                                                                                                                                                                                                                                                                                                                                                                            | 84.0 ± 3.7   | 147.5 ± 3.5 <sup>c</sup>                          |                          |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. 18 days) 40-day-old plants                                                                                                                                                                                                                                                                                                   |              |                                                   |                          |
| Leaf                                                                                                                                                                                                                                                                                                                                                                                            | 128.6 ± 15.0 | 130.3 ± 11.7                                      | 133.6 ± 1.7              |
| Root                                                                                                                                                                                                                                                                                                                                                                                            | 84.3 ± 2.7   | 104.2 ± 5.8 <sup>b</sup>                          | 104.7 ± 1.3 <sup>c</sup> |
| Edible root                                                                                                                                                                                                                                                                                                                                                                                     | 75.2 ± 6.7   | 80.2 ± 1.7                                        | 78.5 ± 1.7               |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. Sofit) 40-day-old plants                                                                                                                                                                                                                                                                                                     |              |                                                   |                          |
| Leaf                                                                                                                                                                                                                                                                                                                                                                                            | 130.3 ± 8.4  | 120.2 ± 3.3                                       | 150.3 ± 3.3 <sup>a</sup> |
| Root                                                                                                                                                                                                                                                                                                                                                                                            | 99.9 ± 1.3   | 102.0 ± 2.7                                       | 121.1 ± 2.8 <sup>c</sup> |
| Edible root                                                                                                                                                                                                                                                                                                                                                                                     | 73.5 ± 3.3   | 73.5 ± 1.7                                        | 86.8 ± 1.7 <sup>a</sup>  |
| <i>Sources:</i> Skugoreva, S.G. and Golovko, T.K., <i>Agrokhimia</i> ( <i>Agrochemistry</i> ), 5, 85, 2007a, in Russian; Skugoreva, S.G. and Golovko, T.K., <i>Agrokhimia</i> ( <i>Agrochemistry</i> ), 2, 66, 2007b, in Russian.                                                                                                                                                               |              |                                                   |                          |
| <i>Notes:</i> Significant differences between the control and Hg-treated plants are indicated by <sup>a</sup> ( $p \leq 0.05$ ), <sup>b</sup> ( $p \leq 0.01$ ), or <sup>c</sup> ( $p \leq 0.001$ ), respectively, $n = 4$ . <sup>d</sup> Hg(NO <sub>3</sub> ) <sub>2</sub> pollution levels (36 and 90 mg Hg kg <sup>-1</sup> DW) correspond to 10 and 25 of MCLs of Hg in soil, respectively. |              |                                                   |                          |

as a metabolite indicator of lipid peroxidation of membranes after HM treatment was found (Tables 27.1 and 27.3). The maximal concentration of MDA in roots of lettuce grown on mercury-polluted soil (36 mg Hg kg<sup>-1</sup>) and in barley roots treated with 100 μM Cd under cool temperature conditions was observed.

**27.5 HEAVY METAL TOLERANCE IN PLANTS**

Plants are able to control the intracellular concentration of HM. Many of them are adapted to elevated HM concentrations due to evolution of tolerance mechanisms. Among the metal tolerance mechanisms in plants, selective metal ions uptake, binding and sequestration of metal cations, metabolic processes changing and engagement of alternative metabolic pathways, metal removal through root secretion, and leaves abscission have been extensively documented (Prasad and Hagemeyer 1999, Hall 2002, Titov et al. 2007). Overall, there are three groups of the tolerance mechanisms: (1) prevention of metal ions inflow to the cell, (2) intracellular tolerance mechanisms, and (3) formation of metal-binding complexes.

### 27.5.1 ROOTS AS A BARRIER ON THE PATH OF METALS TRANSPORT

A first barrier against HM stress operates at the level of root-soil relationship. Root exudates can affect HM, and in particular Cd absorption, by plants through changing the physical and chemical characteristics of rhizosphere. The influence of root exudates on HM bioavailability and toxicity may include modifying the rhizosphere pH, chelating/complexing and deposition with metal ions, and altering the community construction, and the numbers and the activities of rhizospheric microbes (Seregin and Ivanov 2001, Dong et al. 2007).

There is circumstantial evidence that plants can benefit from mycorrhiza colonization on contaminated soils (Colpaert and Vandenkoornhuyse 2001). Mycorrhiza is thought to improve soil nutrition of the plant-host and protect the plant from exposure to metal contaminants by reduced assimilation and/or transfer of metals to the host. The prevention of excess metal uptake might be realized by fungi excretion of metal-immobilizing substances, extracellular sequestration, or well-regulated uptake system coupled to stable plasmalemma.

Roots as the main barrier on the path of metals transport to shoots are able to immobilize HM ions (including Cd and Hg) by means of the cell wall and extracellular carbohydrates (Sanità di Toppi and Gabrielli 1999, Seregin and Ivanov 2001). HM ions seem to be mostly bound by pectic sites and hystidyl groups of the cell wall. The importance of Cd binding to cell walls and the limitation of its subsequent translocation to shoots has been demonstrated for root cells of hyperaccumulators and non-hyperaccumulating plants (Shevyakova et al. 2003, Cosio et al. 2005).

Under low concentrations of HM in the medium, the metal ions enter the roots and reach the xylem through an apoplastic pathway where they can be complexed by several ligands (Sanità di Toppi and Gabrielli 1999, Seregin and Ivanov 2001). Casparian band and plasma membrane of endodermal cells can hold the ions to a certain metal concentration in tissue.

Roots transport HMs across their plasma membrane either by diffusion (mostly by exchange absorption) or by active transport via cation channels or carrier proteins (Costa and Morel 1994, Hall and Williams 2003). These transport mechanisms are believed to be typical for all metal ions, including Cd, Hg, Zn, Cu, Pb, etc. However, Hg<sup>2+</sup> is thought to enter the root cells only after disturbance of plasma membrane sites by binding sulfhydryl groups of membrane proteins. Organic lipophilic mercury compounds move across lipidic phase of plasma membrane readily (Trakhtenberg and Korshun 1990).

In our experiments, the HM content in plant roots was higher than that in shoots and soil. Under high mercury soil pollution, the metal content in aboveground part and in roots increased (Table 27.4). It has been also demonstrated that at cool temperature regime and high Cd concentration (100 μM) in nutrient solution, the root barriers lost their function, and cadmium was translocated to shoots: the Cd content in the shoots sharply increased (by four times) (Table 27.1).

### 27.5.2 ANTIOXIDANT SYSTEM OF PLANT CELLS

At high metal concentration in medium, the barriers and sites of metal ions binding in roots are not sufficient to prevent transport of HM to the shoots. Therefore, oxidative stress in cells can be developed. Antioxidant defense system plays an important role in detoxifying ROS. The antioxidative machinery is antioxidative enzymes and nonenzymatic antioxidants (Dietz et al. 1999). The most prominent antioxidative enzymes are the ROS-detoxifying superoxide dismutases (SOD), catalases, and a large number of H<sub>2</sub>O<sub>2</sub>-reducing peroxidases. The important nonenzymatic antioxidants are glutathione, ascorbate, carotenoids, flavonoids, tocopherol, aromatic hydroxy-acids, anthocyanins, polyamines, polyphenols, and thiols; some of them are regenerated by antioxidative enzymes.

Enhanced activities of ascorbate peroxidase, catalase, and glutathione reductase in two mustard (*Brassica juncea* L.) cultivars alleviated Cd stress and protected the photosynthetic activity (Mobin and Khan 2007). Cd<sup>2+</sup> in concentrations of 0.01, 0.1, and 1 mM stimulated SOD activity and glutathione reductase in pea leaves, but ascorbate peroxidase activity increased only

**TABLE 27.4**  
**Hg Content in the Organs of Some Plant Species under**  
**Different Hg(NO<sub>3</sub>)<sub>2</sub> Soil Pollution**

|                                                                                               |             | Hg Treatments (mg kg <sup>-1</sup> ) <sup>d</sup> |                         |
|-----------------------------------------------------------------------------------------------|-------------|---------------------------------------------------|-------------------------|
| Species                                                                                       | Control     | 36                                                | 90                      |
| Field pea ( <i>Pisum arvense</i> L., cv. Nadezhda) 15-day-old plants                          |             |                                                   |                         |
| Root                                                                                          | 3.4 ± 0.3   | 399 ± 41 <sup>b</sup>                             | 1116 ± 89 <sup>b</sup>  |
| Shoot                                                                                         | 6.6 ± 0.6   | 45 ± 0.1 <sup>c</sup>                             | 108 ± 9 <sup>c</sup>    |
| 40-day-old plants                                                                             |             |                                                   |                         |
| Root                                                                                          | 4.6 ± 0.2   | 289 ± 4 <sup>c</sup>                              | 1180 ± 110 <sup>b</sup> |
| Shoot                                                                                         | 4.0 ± 0.6   | 9.2 ± 1.3 <sup>a</sup>                            | 21 ± 3 <sup>a</sup>     |
| Leaf                                                                                          | 2.4 ± 0.1   | 2.8 ± 0.2                                         | 7.3 ± 0.1 <sup>c</sup>  |
| Stem                                                                                          | 1.8 ± 0.1   | 3.4 ± 0.1 <sup>b</sup>                            | 11 ± 1 <sup>b</sup>     |
| Garden-cress ( <i>Lepidium sativum</i> L.) 20-day-old plants                                  |             |                                                   |                         |
| Root                                                                                          | 2.2 ± 0.3   | 43 ± 1.8 <sup>c</sup>                             | 257 ± 8 <sup>c</sup>    |
| Shoot                                                                                         | 1.6 ± 0.1   | 3.8 ± 0.1 <sup>a</sup>                            | 16 ± 3 <sup>a</sup>     |
| Stem                                                                                          | 1.9 ± 0.3   | 2.1 ± 0.6                                         | 5.2 ± 0.2 <sup>b</sup>  |
| 30-day-old plants                                                                             |             |                                                   |                         |
| Root                                                                                          | 17 ± 1      | 245 ± 5 <sup>c</sup>                              | 1670 ± 36 <sup>c</sup>  |
| Leaf                                                                                          | 25 ± 1      | 15 ± 2 <sup>a</sup>                               | 62 ± 7 <sup>a</sup>     |
| Stem                                                                                          | 20 ± 1      | 14 ± 2 <sup>a</sup>                               | 24 ± 1 <sup>a</sup>     |
| Lettuce ( <i>Lactuca sativa</i> L., cv. Moskovskii parnikovyi) 50-day-old plants              |             |                                                   |                         |
| Root                                                                                          | 14 ± 1      | 65 ± 3 <sup>b</sup>                               | Plant death             |
| Shoot                                                                                         | 4.4 ± 0.1   | 10.1 ± 0.5 <sup>c</sup>                           |                         |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. 18 days) 40-day-old plants |             |                                                   |                         |
| Root                                                                                          | 5.2 ± 0.7   | 48 ± 1 <sup>c</sup>                               | 312 ± 17 <sup>b</sup>   |
| Edible root                                                                                   | 1.9 ± 0.1   | 7 ± 0.1 <sup>c</sup>                              | 18 ± 2 <sup>b</sup>     |
| Leaf                                                                                          | 2.4 ± 0.1   | 16 ± 1 <sup>b</sup>                               | 28 ± 2 <sup>b</sup>     |
| Stem                                                                                          | 2.1 ± 0.4   | 4.5 ± 0.7 <sup>a</sup>                            | 4.8 ± 0.1 <sup>b</sup>  |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. Sofit) 40-day-old plants   |             |                                                   |                         |
| Root                                                                                          | 5.0 ± 0.1   | 10.7 ± 0.2 <sup>c</sup>                           | 498 ± 30 <sup>b</sup>   |
| Edible root                                                                                   | 1.62 ± 0.03 | 6.50 ± 0.03 <sup>c</sup>                          | 26 ± 4 <sup>b</sup>     |
| Leaf                                                                                          | 2.4 ± 0.1   | 8.1 ± 0.6 <sup>b</sup>                            | 33 ± 3 <sup>b</sup>     |
| Stem                                                                                          | 1.5 ± 0.1   | 4.90 ± 0.02 <sup>c</sup>                          | 26 ± 2 <sup>b</sup>     |

*Sources:* Skugoreva, S.G. and Golovko, T.K., *Agrokhimia (Agrochemistry)*, 5, 85, 2007a, in Russian; Skugoreva, S.G. and Golovko, T.K., *Agrokhimia (Agrochemistry)*, 2, 66, 2007b, in Russian.

*Notes:* Significant differences between the control and Hg-treated plants are indicated by <sup>a</sup> ( $p \leq 0.05$ ), <sup>b</sup> ( $p \leq 0.01$ ), or <sup>c</sup> ( $p \leq 0.001$ ), respectively,  $n = 4$ . <sup>d</sup> Hg(NO<sub>3</sub>)<sub>2</sub> pollution levels (36 and 90 mg Hg kg<sup>-1</sup> DW) correspond to 10 and 25 of MCLs of Hg in soil, respectively.

**TABLE 27.5**  
**Peroxidase Activity in the Organs of Some Plant Species**  
**(mL 0.01 n I<sub>2</sub> g<sup>-1</sup> FW) under Different Hg(NO<sub>3</sub>)<sub>2</sub> Soil**  
**Pollution**

| Species                                                                                       | Control     | Hg Treatments (mg kg <sup>-1</sup> ) <sup>d</sup> |                          |
|-----------------------------------------------------------------------------------------------|-------------|---------------------------------------------------|--------------------------|
|                                                                                               |             | 36                                                | 90                       |
| Garden-cress ( <i>Lepidium sativum</i> L.) 30-day-old plants                                  |             |                                                   |                          |
| Leaf                                                                                          | 6.58 ± 0.41 | 6.67 ± 0.43                                       | 7.10 ± 0.43              |
| Root                                                                                          | 18.3 ± 0.5  | 33.8 ± 0.6 <sup>c</sup>                           | 19.40 ± 0.86             |
| Lettuce ( <i>Lactuca sativa</i> L., cv. Moskovskii parnikovyi) 50-day-old plants              |             |                                                   |                          |
| Leaf                                                                                          | 4.19 ± 0.40 | 147.5 ± 3.5                                       | Plant death              |
| Root                                                                                          | 5.59 ± 0.50 | 7.48 ± 0.10 <sup>a</sup>                          |                          |
| Garden radish ( <i>Raphanus sativus</i> var. <i>radicula</i> , cv. 18 days) 40-day-old plants |             |                                                   |                          |
| Leaf                                                                                          | 8.60 ± 0.30 | 8.31 ± 0.10                                       | 16.4 ± 0.60 <sup>b</sup> |
| Root + edible root                                                                            | 7.68 ± 0.10 | 7.22 ± 0.34                                       | 16.2 ± 0.41 <sup>c</sup> |

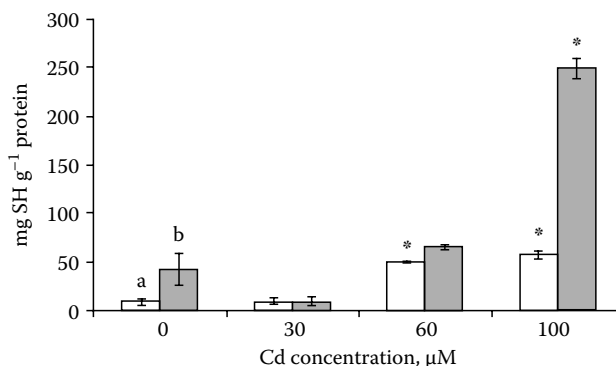
Source: Skugoreva, S.G. and Golovko, T.K., *Agrokhimia* (Agrochemistry), 5, 85, 2007a, in Russian.

Notes: Significant differences between the control and Hg-treated plants are indicated by <sup>a</sup> ( $p \leq 0.05$ ), <sup>b</sup> ( $p \leq 0.01$ ), or <sup>c</sup> ( $p \leq 0.001$ ), respectively,  $n = 4$ . <sup>d</sup> Hg(NO<sub>3</sub>)<sub>2</sub> pollution levels (36 and 90 mg Hg kg<sup>-1</sup> dry weight) correspond to 10 and 25 of MCLs of Hg in soil, respectively.

under high cadmium concentration, peroxidase activity—under low Cd concentration in medium (Balakhnina et al. 2005). Peroxidase activity in many plants is found to increase under mercury stress (Siegel et al., 1987). We also found peroxidase activation in roots of the plants grown in soil substrate containing 36 mg Hg kg<sup>-1</sup> (Table 27.5). Peroxidases are known to catalyze the reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O by oxidizing reduced substances (ascorbate, glutathione, etc.) High Hg concentration inhibited the enzyme activity.

**27.5.3 METAL-BINDING COMPLEXES**

The production of metallothioneins (MTs) and metal-binding complexes is a widespread mechanism of HM detoxification in higher plants. MTs are a family of cycteine-rich, low molecular weight (3.5–14 kDa) proteins. MTs have the capacity to bind such metals as Zn, Cu, Cd, Hg, etc., through the thiol group of its cysteine residues, which represents nearly 30% of its amino acidic residues (Prasad 1999). Phytochelatins (PCs) are  $\gamma$ -glutamyl peptides containing glutamate, cysteine, and glycine. PCs are synthesized from glutathione in the presence of HM by the enzyme phytochelatin synthase (Grill et al. 1985, Prasad 1999). It is known that a very significant role in HM detoxification and tolerance is played by vacuolar compartmentalization, which prevents the free circulation of metal ions in the cytosol and forces them into a limited area. And HM-phytochelatin complexes can transport into vacuole. In the vacuole, because of the acidic pH, these complexes dissociate and HM can be complexed by vacuolar organic acids (citrate, oxalate, malate), and, possibly, by amino acids (Prasad 1999, Sanità di Toppi and Gabrielli 1999, Seregin and Ivanov 2001). Apo-phytochelatins may return to the cytosol, where they can continue to carry out their shuttle role (Sanità di Toppi



**FIGURE 27.4** Content of SH group in soluble protein of 25-day-old barley plants (*Hordeum distichum* L., cv. Novichok) after 6 days of  $\text{CdSO}_4$  treatment. a—leaves, b—roots. Significant differences between the control and Cd-treated plants are indicated by \* ( $p \leq 0.05$ ),  $n = 6$ .

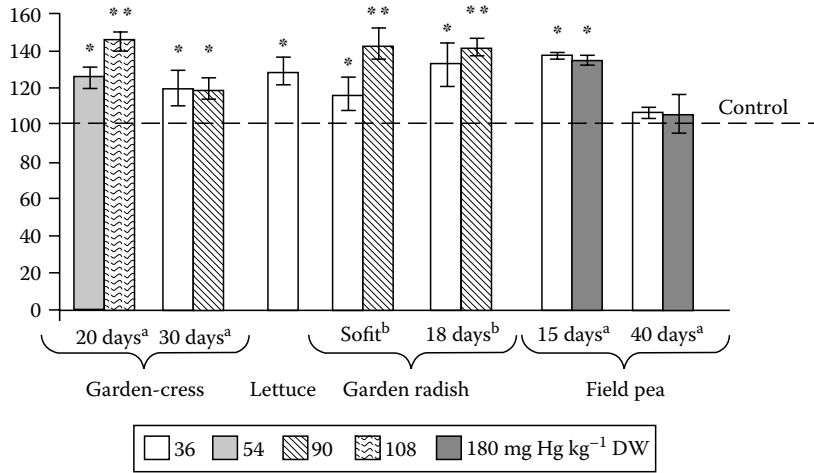
and Gabrielli 1999). Phytoferritin is an iron-protein complex. The protein moiety of ferritin is composed of 24 subunits, which form channels with the deposition of Fe as ferric hydroxyl phosphate (Prasad 1999). The functions of ferritin in plants are storage of Fe and protection of the cell against the toxic effects of free ionic iron. Phytoferritins are usually abundant in roots, root nodules, senescing cells, and seeds. This Fe-binding complex readily meets the demand for iron of the developing chloroplasts.

Overall, metal-binding complexes reduce cytoplasmic toxicity of certain concentrations of HM. They are less toxic to cellular plant metabolism than free metal ions. We found a change of soluble protein structure in barley roots under cadmium impact (Figure 27.4). The amount of the SH groups reacting with HM and detoxifying it was significantly increased compared to the control.

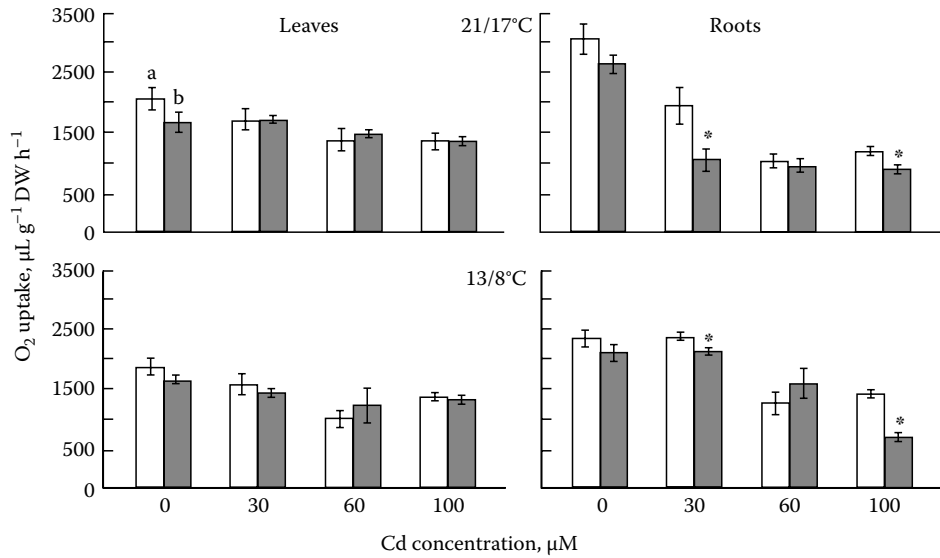
#### 27.5.4 METABOLIC PROCESSES CHANGING

The activation of a repair system and/or changing of metabolic processes can be one of the HM tolerance mechanisms in plants. Respiration is an important plant function, because it is source of energy and intermediates. As process connecting with all sides of plant activity, respiration reflects metabolism state. We found that HMs in moderate concentrations do not change or even increase respiratory capacity. Though, the plant growth was inhibited in mercury stress conditions, respiration rate in plant roots was 20%–40% higher under Cd stress compared to the control (Figure 27.5). It is likely related to the activation of metabolic processes associated with functional cell integrity maintenance and repair of damages (Golovko 1999).

In other experiment, high cadmium concentrations suppressed respiration rate of barley plants, especially in roots, and induced engagement of alternative pathway (AP) respiration (Garmash and Golovko 2009). The contribution of AP increased to 40% of the total respiration (Figure 27.6). At the same time, the lipid peroxidation is intensified, which is an indication of increased oxidative stress (Table 27.1). AP is known to maintain redox balance during mitochondrial electron transport (Millenaar and Lambers 2003) and to alleviate the formation of ROS (Maxwell et al. 1999). A significant AP involving in the roots at low Cd concentration (30 μM) under warm temperature regime, when the respiration rate is relatively high, is evidently required for continuation and active functioning of the citric acid cycle. Organic acids of the cycle are able to bind Cd in vacuoles (Sanità di Toppi and Gabrielli 1999, Seregin and Ivanov 2001). Under high Cd stress (100 μM) and low temperature regime, plant growth was ceased (Table 27.2) and partial death of plants was observed. It is known that during the development of programmed cell death in plants, APs may maintain mitochondrial function after insult with a death-inducing compound or may prevent chronic oxidative stress within the mitochondrion (Robson and Vanlerbergh 2002).



**FIGURE 27.5** Effect of mercury on respiration rate of underground organs in some plant species, % from control values. <sup>a</sup>Days after seedlings appearance, <sup>b</sup>cultivars of garden radish (40 days after seedlings appearance). Mercury was added to a soil in the form of  $\text{Hg}(\text{NO}_3)_2$ . Significant differences between the control and Hg-treated plants are indicated by \* ( $p \leq 0.05$ ),  $n = 5$ . (From Skugoreva, S.G. and Golovko, T.K., *Agrokhimia* (*Agrochemistry*), 5, 85, 2007a, in Russian.)



**FIGURE 27.6** Effect of SHAM, the inhibitor of AOX, on the rate of  $\text{O}_2$  uptake by the organs of 25-day-old barley plants after 6 days of  $\text{CdSO}_4$  treatment under two temperature regimes (day/night). a—control (no inhibitor); b—25 mM SHAM. Significant differences between the control and SHAM-treated samples are indicated by \* ( $p \leq 0.05$ ),  $n = 12$ . (From Garmash, E.V. and Golovko, T.K., *Russ. J. Plant Physiol.*, 56, 343, 2009.)

## 27.6 STRATEGIES IN THE RESPONSE OF PLANTS TO HEAVY METALS

Plants have developed different tolerance strategies to grow on soils rich in HMs (Baker 1981). A large number of tolerant plants are called *excluders*: they are able to restrict root uptake and, in particular, root-to-shoot translocation of HM. These plants have a barrier to avoid uptake of metals. Under high HM levels, the barriers lose their function, and the uptake increases. In other plants, called indicators, passive metal uptake occurs so that the internal concentration reflects the external

level. Indicators have been widely used in the detection of HM impact on the environment. The best phytoindicators are mosses and lichens, but they are not effective for soil biomonitoring. It was found that suitable phytoindicator among angiosperms is dandelion (*Taraxacum officinale* L.) (Bezel' and Zhuikova 2007). Roots and leaves of this plant are able to uptake metals from polluted soil and air, respectively. Dandelion and other species of Asteraceae, which are the most advanced evolutionary plant species and have a wide reaction norm, accumulated more HM such as Cu, Cd, and Co than other plants (Fabaceae, Poaceae) (Bezel' and Zhuikova 2007). The third type of uptake characteristic is plants accumulators. These plants have high accumulation of metals at low external metal concentrations. Accumulators have certain internal mechanisms of HM detoxification, which allow the plant to accumulate high amounts of metals. And some plant species called hyperaccumulators accumulate HMs to exceedingly high levels in the aboveground parts. The term "hyperaccumulator" was introduced by Brooks et al. (1977) for plants that accumulate more than 1 mg Ni per gram of dry weight in their shoots in their natural habitats. To date, more than 400 species of hyperaccumulators of Ni, Co, Mn, Cd, and Zn belonging to 45 families have been identified (Baker et al. 2000). Many of the hyperaccumulators belong to Brassicaceae (Sanità di Toppi et al. 2001).

## 27.7 PHYTOREMEDIATION

Plants concentrate the contaminants within their tissues, thereby reducing the amount of hazardous waste in the environment. This plant property underlies phytoremediation technology (Saxena et al. 1999, Mukherjee 2001). Generally, plant hyperaccumulators are used for phytoremediation, and most of the commonly known hyperaccumulators belong to the Brassicaceae family (Sanità di Toppi et al. 2001). The volume of contaminated plant biomass can be reduced by ashing or composting; the residue should be treated as a hazardous waste or can be recycled for the production of valued metals (Mukherjee 2001). Phytoremediation is cost-effective in comparison with current expensive engineering methods, such as washing or incineration of soils. Phytoremediation is used for remediation of soils and decontamination of air and water. Nowadays, transgenic plants might represent a strong tool for developing effective HM phytoremediation strategies. Pilon-Smits et al. (1999) overexpressed the *E.coli*  $\gamma$ -glutamylcystein synthetase and glutathione synthetase enzymes in *Brassica juncea*. These transgenic plants concentrated more Cd than normal plants in their shoots. It has been reported that a modified bacterial mercuric ion reductase has been introduced into *Arabidopsis thaliana*, which converts  $Hg^{2+}$  into  $Hg^0$  and volatilize Hg in significant quantities (Rugh et al. 1996). Phytovolatilization is a perspective method for removing Hg as well as As and Se from the contaminated soils (Mukherjee 2001).

## 27.8 CONCLUSIONS

The last 10–15 years were marked by considerable progress in studying the problem of plants tolerance and adaptation to HM environment pollution. However, the problem is not completely solved yet. Further production development, the earth population upsurge and resource consuming, contamination of the environment, and accelerating imbalance in nature compel us to take strong measures regarding defense and maintenance of biodiversity. Therefore, extension of knowledge on ecological adaptations of plants is of great importance. Plant responses to an HM depend on genotype tolerance, its actual age, phase of development, life form, climatic and edaphic factors, and finally concentration and exposure time of the metal. In nature, the effects of pollutants on plants are often modified by other existing factors. Plants in areas with high anthropogenic pressure are more sensitive to other abiotic and biotic effects. Different plants tolerances to cadmium and mercury effects caused by the HM uptake and accumulation peculiarities, allocation of the metals to the plant organs, responses of the plant metabolism, detoxification mechanisms, and the plant growth conditions were observed. Mercury in two concentrations in soil (36 and 90 mg Hg kg<sup>-1</sup>)

suppressed growth of plants. The most adverse effect of the metal was found on lettuce (*Lactuca sativa* L., cv. Moskovskii parnikovyi) growth. This cultivar can be recommended as phytoindicator for biomonitoring mercury-polluted soil. It was also shown that the cool temperature regime impaired the growth of the barley plants treated by cadmium. Alternative respiratory pathway is believed to be one of the mechanisms for maintenance of the root cell homeostasis under cadmium-induced stress. Knowledge on plant responses to HM effects is attractive to the further development of phytoremediation, development of genotypes and phytocenoses tolerant to HM, and efficient use of natural resources.

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# *Part V*

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*Plant and Crop Responses  
to Weeds, Pests, Pathogens,  
and Agrichemical Stress Conditions*

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# 28 Stress in Plants and Crops Induced by Parasitic Weeds

*Andrea Cavaliere and Asghar Heydari*

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## 28.1 INTRODUCTION

The story of agriculture is mainly the story of weed interference. Weeds are considered one of the main problems in agriculture and are responsible for large yield losses in various crops every year (Lampkin, 1990; Bhuler, 1996; Anaya, 1999). Their effect on the agricultural production can be quantifiable to a loss of one-third of the total production, due to competitive factors (Oerke et al., 1994). Plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. Stress is often defined as any factor that decreases plant growth and reproduction below the potential of the genotype (Osmond et al., 1987). However, biotic stress remains a broadly defined and poorly understood form of plant stress, partly because its application is difficult to control, and partly because its physiological consequences are often highly variable (Ayres, 1992). To survive under such conditions, plants have evolved complex mechanisms to perceive external signals, allowing optimal response to environmental conditions (Fujita et al., 2006). For example, plants compete with each other for light, water, and nutrient elements; therefore, the potential inherent ability to directly inhibit competitors is their strategy to survive. Competition takes place in optimal habitats; therefore, plants or even groups of plants have to move to less favorable and sparsely populated habitats. Movement will only be successful if nutrition can be obtained elsewhere. While the vast majority of plants and weeds are autotrophic where nutrition is guaranteed by the formation of leaves and roots, there are heterotrophic plants, such as parasitic ones, where nutrition is guaranteed by their host. Parasitic weeds are serious pests in agricultural fields and pose a tremendous threat to world agriculture, mainly because they are at present almost uncontrollable. Parasitic plants have lost their autotrophic way of life during their development process. The system is made up of the coexistence of two different organisms, which supports one of them (parasite) on the expense of the other (host). The host through leaves can synthesize organic substance and photosynthesize (chlorophyll + solar energy), while through roots, it can synthesize inorganic substance and the uptake of water and minerals. On the other hand, the parasite obtains carbohydrates from another organism. Hence, they are directly or indirectly dependent on autotrophic plants for carbohydrates, nitrogen,

water, etc. Usually, the host differs from the total number of different species that can be parasitized, and is called “host range,” while it becomes “host preference” when it develops into the most desirable host for optimal growth. Parasites acquired crops as their food resource, enabling them to survive in large numbers even on nutrient-poor sites, since parasitism allows plants to access a rich hydroponic nutrient source. Evolution is opportunistic and parasites are the greatest opportunists. Parasitic weeds are serious pests in agricultural fields; they are a taxonomically diverse group of angiosperms that rely partially or completely on host plants for carbon, nutrients, and water. Root-parasitizing weeds are of economic importance because they reduce crop yield and quality. The parasites remove water, minerals, and photosynthates from the crop, reducing the host’s ability to grow and develop. Crops that are parasitized usually grow more slowly, and, depending on the severity of infestation and the parasite, biomass production is lowered or the host is almost killed. For decades it was assumed that improved breeding and agronomy could overcome the parasites, and little effort was invested in understanding the nature of parasitism. Because of the lack of control methods at the time of attachment, the main means of controlling parasitic weeds had focused on the varying levels of host tolerance and on reducing the soil seed bank, preventing seed set and inhibiting spread from infested to noninfested areas (Boari and Vurro, 2004; Vurro et al., 2009).

## 28.2 TYPES OF PARASITIC PLANTS

Parasitic weeds are generally divided into two major categories (as root and stem parasites, according to the plant organ they attach to), but can be classified in a broader way as follows:

- Parasite: a plant depending on another plant for part or all of its nutrition
- Hemi-parasite or semi-parasite: a plant that is only partially parasitic, possessing its own chlorophyll and photosynthetic ability (may be facultative or obligate)
- Holoparasite: a plant that is totally parasitic, lacking chlorophyll, and thus unable to synthesize organic carbon
- Obligate parasite: a plant that cannot establish and develop independently
- Facultative parasite: a plant that can establish and grow independently, but normally behaves as a parasite to obtain some of its nutrition

Parasitic plants are defined by the production of specialized feeding structures, described as haustorium. The haustorium is the key organ in all parasitic plants, physically and physiologically bridging the parasite and the host. Nutrients and water are transferred through the haustorium from the conductive system of the host into that of the parasite, and all hormonal interactions between the two organisms are facilitated by this organ. The haustorium develops when intrusive cells of the parasite penetrate host tissues, reaching the vascular system of the host (Smith et al., 2001). Parasites become established via germination. Seeds land on the host tissue and germinate after reaching a chemical stimulus from the host. A modified lateral root becomes a haustorium; this root is chemotrophic, that is, responding to a chemical gradient, and contacts the host epidermis. The root then attaches by pushing against the plant and forming a disc, called a hapteron, and secretes a polysaccharide adhesive. The root tip then mechanically penetrates the host, apparently without enzymatic digestion, and establishes a vascular connection by attaching vessels and positioning phloem next to the leaky host phloem. Mistletoes were formerly alleged to receive no host carbohydrates, but a substantial heterotrophic carbon gain has been measured in mistletoes, even without phloem connections. A direct xylem-to-xylem continuity between host and parasite is not easy to demonstrate. Mistletoes often exhibit high transpiration rates during the day, through stomates and cracks in the epidermis. Losing substantial water from the leaves and stems of the parasite results in a steep water potential gradient, favorable to drawing water into the mistletoe plant. Nitrogen is supplied to the parasite in the xylem stream, and the high transpiration rates; hence, high water demands appear instead to represent a nitrogen-gathering mechanism for the mistletoe. Typical thick, fleshy root parasites

generally lack any adaptations to restrict water loss from their achlorophyllous stems and leaves because they tend to lack the waxy coating, cuticle. Dodder and mistletoes are serious problems for plants. Dodder is weedy and can cover woody plants and damage certain economically important crop plants. Mistletoe can become so abundant on a tree that most of the foliage is of the parasite, not of the host. In general, experts generally state that parasitic plants rarely, perhaps never, kill the host plant, so that the host and parasite live unhappily together in some balance.

### 28.2.1 IMPORTANT PARASITIC WEED SPECIES

The economically important parasitic weed species derive from three families:

- Orobanchaceae: *Orobanche*
- Scrophulariaceae: *Striga*, *Alectra*
- Cuscutaceae: *Cuscuta*

#### 28.2.1.1 Orobanchaceae

According to the occurrence of *Orobanche* spp., the major problems with these weeds are found around the Mediterranean Sea that cause major yield reductions in crops. Yield reduction is dependent on the coincidence and the severity of attack, with yield losses from 5% to 100%. *Orobanche* spp. are essentially restricted to dicotyledonous hosts. Species of the family *Asteraceae* are preferred by *Orobanche*, which, in fact, provide about 150 hosts parasitized by 59 *Orobanche* species.

The genus *Orobanche*, as currently classified, contains over 100 species of obligate root holoparasites in both the Old and New Worlds. These plants are known by the English name “broomrape” because they were thought to grow as tubers (“rapum”) from brooms (the common name for the legume *Cytisus*). The genus reaches its greatest diversity in the Mediterranean climates and in Western Asia. Most of the economically important pathogens are species of the Old World. The major crop hosts for *Orobanche* are legumes, solanaceous crops (eggplant, tomato, tobacco, and potato, but not *Capsicum* peppers), umbels (carrot, parsley, and celery), cole crops (cabbage and cauliflower), lettuce, and sunflower. Control is difficult due to seed longevity in the soil (more than five decades), small seed size (less than the width of a human hair), fecundity (thousands of seeds per plant), and a subterranean phase (seeds germinate beneath the soil and parasitize the host before they emerge and appear). Broomrapes have their greatest impact in the Balkans, the Nile Valley, Central Asia, southern India, and Nepal. Damage varies with the level of infestation, and total crop failures have occurred in some cases. There have been numerous studies of the host range of *Orobanche* species. It has been shown that *Orobanche ramosa* can parasitize plants from 11 different dicot families, in fact, more different hosts than any other broomrape. Major agronomically important hosts include solanaceous crops, cabbage, cauliflower, hemp, carrots, lettuce, and some legumes. The related species *O. aegyptiaca* causes especially severe damage to melons in Central Asia, where broomrape not only reduces the yield and weakens the melons, but also induces the production of a toxin within the melons that renders them unmarketable.

*O. aegyptiaca* and *O. ramosa* attack especially Solanaceae, like eggplant, potato, tobacco, tomato, etc. Grain and fodder legumes are parasitized by *O. crenata* and *O. minor*, while *O. cumana* threatens sunflower. The most damaging species in legume crops is *O. crenata*, occurring mainly in the Mediterranean region. The most important legume hosts are faba bean, lentil, pea, chickpea, and common vetch. Serious damage in faba bean occurs, for example, in Egypt, Morocco, and Spain.

*Orobanche aegyptiaca*, although more widespread than *O. crenata*, is of less importance in legumes due to the different temperature requirements. This species requires higher temperatures for germination and development. Therefore, it is more often found parasitizing summer crops, like tobacco or tomato, instead of the legumes grown during winter.

Although *Orobanche* is spread all over the world, losses occur mainly in South and East Europe, North Africa, and West Asia. Parasitism can cause huge or limited yield reduction depending on the number of parasite attachments per host plant and the time of infestation. In some cases, farmers have to abandon their fields. During the period 1968–1978, the faba bean production area in Egypt dropped down by 29% because of heavy infestation with *O. crenata*. In Yugoslavia, the production area for sunflowers decreased by 37% in the 1950s (Nickrent and Musselman, 2004). The parasite could be not forced back until resistant varieties were grown.

Damage by the parasite is dependent on the infestation level in the field, that is, the probability of the host root to meet the seeds of a parasitic weed. Yield loss is also dependent on the time of infestation and, therefore, on the duration of infection. Most of the attached parasites are not visible aboveground, since usually only about 1%–30% of the attached plants emerge.

#### 28.2.1.2 Scrophulariaceae

*Striga* is at the moment the biggest biological hindrance in grain and corn production in Africa. Three species are seriously damaging: *Striga asiatica* and *Striga hermonthica* on cereals, and *Striga gesnerioides* on legumes like cowpea, groundnut, and bambaranut. *Striga asiatica* and *Striga hermonthica* are almost entirely specific to grasses, while *Striga gesnerioides* prefers dicot host. The most important species from the economical viewpoint is *Striga hermonthica*, attacking sorghum and millet crops in the Sahelian area. Crop losses due to *Striga* were estimated to more than \$7 billion. The occurrence of the economic importance of *Striga* species is reported in 59 countries, especially in east and west Africa and Asia. Witchweeds (*Striga* spp.) have a greater impact on humans worldwide than any other parasitic plants, because their hosts are subsistence crops grown widely in Africa and Asia. Such crops include maize, sorghum, pearl millet, finger millet, rice, as well as sugarcane and legume crops, such as cowpea and groundnut. The name “witchweed” derives from the effect these parasites have on their host, in which most damage occurs before the parasite is visible aboveground. This “bewitching” behavior is also reflected in the Latin name, which means “hag” or “witch.”

*Striga* is an obligate hemiparasite that reaches its greatest diversity in the grasslands of Africa, although it also occurs in India, the Far East, and Australia. Two species, *S. asiatica* and *S. hermonthica* cause the most damage to crops worldwide. Most of the *Striga* species have complex life cycles. Several discrete steps can be recognized: diaspore, after ripening, conditioning, haustorial induction, attachment, penetration, seedling development, emergence, and flowering.

The genus *Alectra* spp. (hemiparasitic) comprises about 30 species. Four are reported to be significant pests. The most important is *Alectra vogelii*, parasitizing mainly cowpea, bambara, and groundnut. Excessive damage is caused by *Alectra picta*, attacking predominantly *Vigna unguiculata* (cowpea), a food legume of the savannah areas in Africa. *Alectra orobanchoides* can be a problem in *Nicotiana tabacum* (tobacco) in South Africa, while *Alectra fluminensis* has been described as a pest in *Saccharum officinarum* (sugarcane) in Latin America.

#### 28.2.1.3 Cuscutaceae

*Cuscuta* spp., or dodder species, are among the best known of all parasitic plants. The biology and control of dodders has been reviewed in Dawson et al. (1994). Dodders have a broad host range, although monocots are less preferred. The genus *Cuscuta* contains three subgenera. The first subgenus, *Monogyna*, are robust vines that may attack and kill fruit trees, the second subgenus, *Cuscuta*, are more delicate in structure and favor herbaceous hosts, the third subgenus, *Grammica*, have very low host specificity and are easily found parasitizing different host species simultaneously (Nickrent and Musselman, 2004). Although dicots are preferred, attack on monocots has been observed. Dodders may be the most important parasitic weeds of legumes in temperate regions. Of particular importance is *Cuscuta campestris* on alfalfa (*Medicago sativa*). The alfalfa and dodder seeds are similar in size, and so the parasite is spread with the host. The wide range of hosts attacked by dodders is reviewed in Dawson et al. (1994). The most effective means of control is seed sanitation.



Because the surface of dodder seeds is minutely roughened, dodder seeds stick to felt rollers, while alfalfa seeds pass over. Dawson et al. (1994) also reviewed several herbicide treatments that are directed at the newly germinated seeds of dodder.

### 28.3 METABOLIC PATHWAY

The germination of parasitic plants represents a pivotal role in their metabolic pathways. This process is unique because it depends on the reception of chemical stimulus. A typical life cycle is shown in Figure 28.1. The seeds of parasitic weeds buried in the soil become sensitive to germination-stimulating signals from host roots. Usually, as soon as a crop is planted, the roots of crop plants produce germination stimulants and induce the germination of part of the parasitic weed seed population (Bouwmeester et al., 2003). In addition, it is widely reported that, following imbibitions of the seeds, and before chemical stimulation, a wet environment is required for several days in suitable temperatures to render the imbibed seeds responsive to germination stimulants and to allow germination (Joel, 2000). Seeds of most parasitic plants will readily germinate only if the appropriate environmental conditions with respect to water, oxygen, temperature, and light are met (Boone et al., 1995; Estabrook and Yoder, 1998; Sauberon et al., 2007). This preparatory phase is termed “conditioning” or “preconditioning.” After this stage, the radicle of the parasite grows to the host root. Haustorium-inducing factors are produced by the host root and a haustorium is formed. A xylem connection is established and the parasitic plant emerges. A large part of the damage to the host is inflicted before the emergence of the parasite. The parasitic plant reaches maturity and produces flowers and seeds. The seeds end up in the soil seed bank where they gradually become sensitive to germination signals. Seeds of parasitic weeds are buried in the soil and are subsequently “preconditioned” (Boone et al., 1995; Joel, 2000; Bouwmeester et al., 2003). Many studies on seed conditioning have tried to explain such a pathway, but no single research explains whether any of these molecular and metabolic developments correspond to the receptivity of germination stimulation. It has been speculated that conditioning may allow the leaching of germination inhibitors from the seed, or that conditioning may increase the permeability of the seed coat or other seed structures, or that structural modifications occurring in the seeds allow the stimulant to access its putative cellular target (Godwin et al., 1998). Parasitic plants use host molecules to trigger developmental

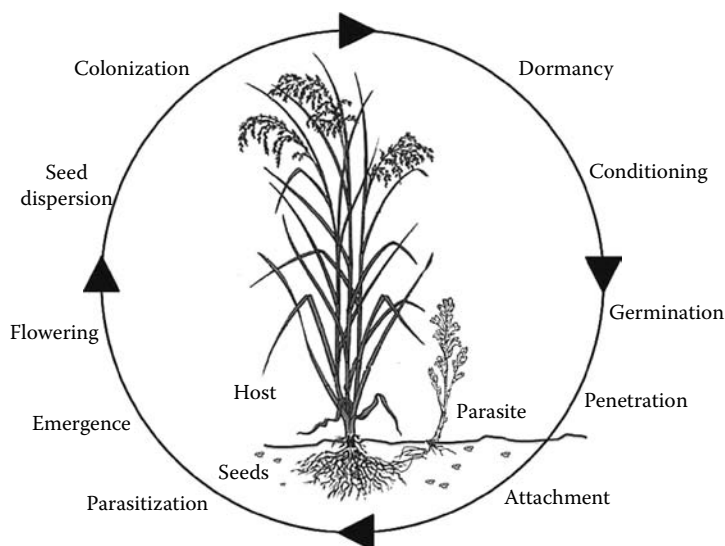


FIGURE 28.1 Life cycle of a parasitic plant.

programs essential for parasitism. One such program rules the development, growth, and role of haustoria. The haustoria is a parasite-specific organ responsible for the attachment and invasion of host tissues that attach, attack, and act as physiological conduits for robbing the host plant of water, minerals, and carbohydrates (Kuijt, 1969; Musselman and Dickison, 1975). All of the information necessary to define plant ontogeny is established within the meristem, a space of just a few millimeters. Haustoria develop from changes in the growth and development of the meristem in response to external stimuli. Plant apical meristems consist of an incessant embryonic tissue maintaining root or shoot elongation. In some parasitic species, primary haustoria develop as a transformation of root apices (Kuijt, 1969). In others, secondary haustoria develop from parenchymal cortical cells near the root tip as well as at more proximal positions along the root (Baird and Riopel, 1983; Estabrook and Yoder, 1998). The attachment of the parasite to the host is facilitated by mucilaginous substances produced by haustorial hairs. Attachment is not discriminatory and can occur on plastic or string as readily as host roots. The epidermal cells that overlay the swollen root cortex develop long haustorial hairs that function in host attachment (Baird and Riopel, 1983). After the haustorium initiation, cortical cells differentiate into vessel elements that form a xylem bridge between the host and the parasite. Haustorium development is induced in the roots of parasitic plants by molecules exuded from host roots. In contrast, the development of vessel members requires a direct contact with the host tissues. Therefore, it is likely that at least two deferent types of host signals are necessary for haustoria to mature. Even though a large number of host species can be parasitized, only a subset is selected when the parasite is presented with a choice (Atsatt and Strong, 1970; Werth and Riopel, 1979; Gibson and Watkinson, 1991). Host selection presumably allows the parasite to maximize the number of beneficial associations, such as those with nitrogen-rich legumes. Legumes, in fact, are often favored because the parasite's mineral requirements are met in part through connections with the host (Visser et al., 1990; Seel et al., 1993; Press, 1995). On the other hand, host selection allows the parasite to minimize nonproductive associations, such as those between roots of the same individual or between roots of closely related individuals (Yoder, 1997). A clear example of this is the avoidance of self-parasitism by most parasitic plants. Some parasites, like *Striga*, completely lack haustoria in the absence of host plants. Haustoria may be produced by mono- or dikaryotic mycelium of rust fungi. Monokaryotic haustoria (M-haustoria) merely appear as intracellular extensions of intercellular hyphae with no significant morphological specialization (Voegelé and Mendgen, 2003). Dikaryotic haustoria develop from external haustorial mother cells (HMC) with a slim neck that penetrates into the host cell and a haustorial body that forms distally to the neck (Voegelé and Mendgen, 2003). Penetration generally is mediated by a combination of intrusive growth and enzymatic digestion (Kuijt, 1969). The evidence for intrusive, mechanical penetration comes from the appearance of crushed host cells at the site of haustoria entry. Upon infection, increased turgor pressure at the tip of the appressorium allows it to mechanically penetrate the cuticle and host cell walls (Estabrook and Yoder, 1998). The HMC, therefore, functionally resemble an appressorium. However, it remains to be elucidated if the functional similarity extends to the molecular level. The expanding haustorium invaginates the host plasma membrane and a new membrane is probably synthesized. Therefore, haustoria are not truly intracellular. They remain outside the physiological barrier of the host cell. With the formation of the haustorial body, a zone of separation between the plasma membranes of the host and the parasite is established. It consists of the fungal cell wall and the extrahaustorial matrix. The extrahaustorial matrix resembles an amorphous mixture of components, mainly carbohydrates and proteins, partly of fungal, but primarily of plant origin (Harder and Chong, 1991). The initial biotrophic phase is characterized by the presence of an interfacial matrix separating host and parasite plasma membranes (Perfect and Green, 2001). Upon the switch to necrotrophic growth, the host plasma membrane surrounding the hyphae disintegrates and parasitic growth continues with narrower unsheathed hyphae. It, therefore, seems likely that this zone of separation plays an important role in the

maintenance of the biotrophic lifestyle. Undoubtedly, the extrahaustorial matrix represents a formidable trading place for the exchange of nutrients and information between the host and the parasite (Heath and Skalamera, 1997).

## 28.4 DAMAGE

The effects that parasitic plants have on host plants are diverse. The acquisition of host resources can exert strong effects on host growth, allometry, reproduction, and physiology (Press et al., 1999). They can lower host growth rate and reproductive output, change host architecture, and even influence host interactions with other organisms, such as mycorrhizae, pathogens, herbivores, and pollinators. Generally, parasitism reduces host productivity and/or reproductive effort, either in root parasites (Matthies, 1995, 1996, 1997; Seel and Press, 1996; Davies and Graves, 1998; Matthies and Egli, 1999) or shoot parasites (Jeschke et al., 1994a,b; Silva and Martínez del Río, 1996; Tennakoon and Pate, 1996; Howell and Mathiasen, 2004). The presence of a parasite usually alters drastically the host metabolic functions, such as photosynthesis, respiration, and uptake of water and solutions. The damage generated by the parasite in such circumstances is more important than direct competition for resources. For example, host productivity can be brutally reduced by parasite-induced change in CO<sub>2</sub> exchange, or loss of water from the host may lead to a decrease in the host stomatal conductance and, consequently, a fall in the rate of host photosynthesis. Basically, a parasite affects host performance by abstracting resources that are essential for the subsistence of the host. Host-derived materials may be transferred from source (crop) to sink (parasite) through straw-like penetrations, called oscula, into the host vascular system (Dörr, 1997). Hence, competition between host and parasite takes place for water, inorganic nutrients, and organic compounds. The level of injury generated by the parasite depends not only on how much the resources are removed but also on the supply available to the host, which may be constrained by the environmental conditions. In most cases, the reduction in host performance is significant, and in the most extreme cases, parasitism may result in host death (Aukema, 2003). Significantly, for community-level impacts, effects on the host are often disproportionately great in comparison to the size of the parasite. This can result from both inefficient use of the resources by the parasite, such that reduction in host biomass is generally greater than the increase in parasite biomass (Matthies, 1995, 1996, 1997; Marvier, 1998; Matthies and Egli, 1999), and from impacts on host physiology that further impair host performance (Ehleringer et al., 1986; Watling and Press, 2001). Further impacts can occur through effects on the host allometry and architecture (Ehleringer et al., 1986; Wanner and Tinnin, 1986; Parker and Riches, 1993; Sala et al., 2001; Meinzer et al., 2004). Parasites can affect host photosynthesis in a variety of ways and at a range of scales, acting at either the leaf and/or the whole-plant level. The magnitude of the impact varies with species, both host and parasite, and ranges from tiny to cruel. Any mechanism that lowers the host photosynthetic rate will also increase the chance of photoinhibition occurring in infected plants. The reduction in host biomass is not explained simply by source–sink relationships, but by phytotoxic effects that change the partitioning of the host photosynthate from shoot to root and an overall reduction in the photosynthetic rate (Ransom et al., 1996). Many species also cause a shortening of the internodes in infected hosts, which causes increased self-shading and, thus, a reduction in light interception (Press et al., 1999). In addition to the injurious effects that some parasitic plants have on host photosynthesis at the leaf level, some are also able to impact negatively on whole-plant carbon fixation through the effect they have on host architecture. Graves et al. (1989), in a carbon budget model, estimated that over 80% of the reduction in host growth could be attributed to the reduction in photosynthesis, while Cechin and Press (1993) demonstrated that the effect on photosynthesis was generated critically by the deficiency in nitrogen. Anyway, the degree of pathogenicity exhibited by parasitic plants is great, from those that exert little impact on their hosts to those that dramatically affect the host physiology and fecundity. Among factors affecting pathogenicity are the biomass ratio of parasite to host, the number of parasites attached to an individual host plant, and the length of time required for the parasite to complete its life cycle.

Even though there can be dissimilarity in pathogenicity and life cycle dynamics, all parasitic plant species have evolved under the condition that they do not kill their hosts prior to successful reproduction.

## 28.5 CONCLUSION

The changing agroclimatic conditions have boosted the infestation and proliferation of the parasitic weeds at an increasing rate. As a result, a war has been waged against these parasitic plants. Unfortunately, the results are not as estimated. The reason is mainly due to the close affiliation of parasitic plants to their host, their complex life cycle and the longevity of seeds (Joel et al., 2007). All these characteristics have not allowed researches to develop a feasible “parasitic weed control”! For decades it has been assumed that crop breeding and agronomy production could overcome parasitism. This belief have generated little interest in understanding the real nature and physiology of parasitism. Their origin, the evolution from nonparasitic plants, their population structures, their evolutionary pathways toward becoming crop parasites, and their ecology are still unclear (Balázs et al., 2009). In fact, due to the lack of knowledge about parasite weeds, it is still impossible to predict whether the infestation levels will be high or low, showing how much needs to be learnt. What has still to be realized and metabolized by a majority of the people is that parasitic plants are not normal weeds. Parasitic plants are a diverse group of organisms that have evolved in a number of ways. This evolution relies on an incessant parasite dependency on the host, ranging from complete dependency upon host nutrients and carbohydrates to an ability to survive and reproduce in the absence of the host. Hence parasitic plants, which are not in direct contact with the soil and soil factors for their growth, have evolved survival means different from normal green plants. For this reason, contrary to general belief, parasitic plants have all the benefit by not billing their hosts, or at least, prior to successful reproduction. Therefore, it is much more difficult to manage or control parasitic weeds. As native components of the ecosystem that have coevolved with their hosts, management rather than eradication is the only rational approach. Understanding the complex and fascinating biology of parasitic plants will require continued research by those engaged in both basic and applied scientific disciplines. Preventing early growth stages, such as seed germination, host attachment, and tubercle development, could be a strategy to deal successfully with the parasite, bringing about its management. Above all, control methods should focus on reducing the soil seed bank and regulate the parasite’s early developmental stages, as most of the damage to the host is inflicted before parasitic plants emerge above the soil. In this context, natural compounds that inhibit or stimulate seed germination could be attractive and environmentally friendly tools to reach that objective (Boari and Vurro, 2004). Most of the damage to the host is done before the parasitic weed emerges above the soil. To overcome this phase, the approach should be the accomplishment of different methods with more emphasis on those that are particularly addressed to neutralize key processes in the parasite. Such methods should lead to a more rational management strategy able to reduce crop damage and suppress the expansion of a virulent seed bank in the fields. As already highlighted, no conventional control can be exerted due to the intimate relation between the parasite and the host. In fact, when the parasite is observable, injury to the crop has already been accomplished. The ineffectiveness of conventional control methods is due to numerous factors, such as the complex nature of the parasites, that reproduce prolifically, have tiny but long-lived seeds, and are difficult to detect until irremediable injury to the crop has been done. So, the only alternative could be the application of the main principles of parasitic weeds management by means of reducing the soil seed bank, preventing seed set, and constraining the spread from infested to noninfested areas. The close relation between the host and the parasite hinders control such as through the application of herbicides. To be effective, the herbicides should be applied at a stage of the crop when the parasite is still underground. At this stage, the herbicides concentrate in the parasites by translocation through the host plant or through the soil solution until they totally defeat

and die. In point of fact, identifying the timing and the rate of herbicide application is critical because a proper concentration proportional to the parasite biomass has to be achieved without causing damage to the crop. Due to the scalar growth of the parasite and by the presupposition that when the parasite is detectable it is already too late, the herbicide application is not economically effective. Although numerous control measures have been developed in agriculture, only a few are effective due to environmental conditions. One of the most suitable control options could be the development of resistant crop varieties. Unfortunately, in many crops, no resistant varieties have been discovered to date. In fact, as native components of the same ecosystem, parasites and hosts have coevolved in a certain environment through a successful survivor strategy. It looks like the only approach in parasitic weed management could consist of the integration of direct different cultural practices, such as crop and cultivar choice, together with biological control. As in an integrated weed management system (IWMS), a solution to this problem could be combining agronomic choice with biological control. The common practice to tackle weed infestation is to use products obtained by chemical synthesis, although herbicide resistance in weeds is rapidly expanding throughout the world with higher costs of production and greater environmental impact (Anaya, 1999; Lemerle et al. 2001). Inevitably, this practice has caused both ecological and environmental problems and risk to human health (Wyse, 1994; Buhler, 1996; Caporali, 2004). For this reason, in the last years, much emphasis has been put on finding out new and alternative solutions to weed management (Dudai et al., 1999; Cavalieri and Caporali, 2010) and pest control. Research on traditional management of crop pests has mainly focused on a more rational use of natural components and processes of the agroecosystem, providing a chance for more appropriate technologies to be used for a sustainable production without damaging the environment (Anaya, 1999). As a consequence, considerable interest has increased in the development of natural products, plant extracts, and essential oils. Biological control by means of essential oils has been already tested for weed control in contraposition to conventional management, such as synthetic herbicides. Results permitted to assert that essential oils are effective in reducing seed germination in controlled conditions, while environmental factors and enzymatic and microbiological transformations make their application in the open field more difficult (Cavalieri, 2009). Biological control is particularly attractive in suppressing root parasitic weeds in annual crops, because the intimate physiological relationship with their host plants makes it difficult to apply conventional weed control measures. Besides the use of essential oils, a novel approach to increase the level of control is to use a “multiple-pathogen strategy” (Charudattan, 2001). In this strategy, two or more pathogens are combined and applied before or after parasite emergence. The possibility of using fungal toxins as natural herbicides is another alternative (Boari and Vurro, 2004; Vurro et al., 2009). Unfortunately, as already observed for essential oils, many toxins are not selective, as they are able to cause the same toxic effects both on host and on nonhost plants. Further research is necessary to develop an appropriate technology for biological control applications. If properly and successfully developed, the advantages of such tools should lie in their application at a very low rate and concentration. The application could be made when the host plants are already fully developed and vigor, so that they inhibit seed germination of the parasitic plant avoiding any toxic effect to the crop. Biological control, such as the application of toxins or essential oils, is a feasible tool even if it apparently does not seem to be persistent. The low persistence due to their biodegradability and easy catabolization in the environment has to be reconsidered in the scenario of natural products used in some kind of sustainable agriculture, such as organic farming. Vurro et al. (2009), have already demonstrate as the application of fungal toxins as inhibitor of seed germination affects the size and shape of the germ tube, while Zanellato et al. (2010) have demonstrate the genotoxic and phytotoxic effects of essential oils as preemergence bioherbicides. Both the application have the advantage of not being persistent in the environment, and dangerous for the human health. The shortening effect could further protect the development of the host plant from the parasite, reducing and/or defeating the seed germination enough to not be able to reach the root of the host or compete with it.

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# 29 Involvement of Insect Pests in Plant and Crop Stress

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## 29.1 INTRODUCTION

Several stresses can be involved during the life of plants. One of the major stresses is caused by tissue damage. Tissue damage in plants is most often associated with insect herbivore infestation. The insect uses the plant as a source of food but the plant tissue consists of dilute nutrients in a matrix of indigestible structural compounds, such as cellulose and lignin, and a variety of allelochemicals. The insect differs from other animals in that it lacks the capacity to synthesize sterols. The insect, in fact, must extract sterol together with several other essential nutrients (amino acids, carbohydrates, lipids, fatty acid, vitamins, trace element) from their food (Behmer and Nes, 2003). Optimal insect growth, survival, and fecundity require certain proteins: carbohydrate ratios, which may vary considerably among species and developmental stages (Schoonhoven et al., 2005). The food is not totally converted to insect biomass but follow the decrease line from food ingested to growth and

**TABLE 29.1**  
**Average Value of Performance and Indices of Nutritional Utilization by Mandibulate and Haustellate Herbivorous Insects (Range in Parentheses)**

|                                 | AD (%) | ECD (%) | RCR (mg per mg per Day) | RGR (mg per mg per Day) |
|---------------------------------|--------|---------|-------------------------|-------------------------|
| Mandibulate (i.e., Lepidoptera) |        |         |                         |                         |
| Herbs                           | 53     | 41      | 2.0 (0.27–6.0)          | 0.37 (0.03–1.5)         |
| Grasses                         | 43     | 45      | 2.0 (0.07–4.8)          | 0.29 (0.06–0.62)        |
| Tree                            | 39     | 37      | 1.5 (0.31–5.0)          | 0.17 (0.03–0.51)        |
| Haustellate (i.e., Homoptera)   |        |         |                         |                         |
| Herbs                           | 60     | 65      | 1.0 (0.90–1.6)          | 0.39 (0.11–0.67)        |

*Sources:* Slansky, F. and Scriber, J.M., Food consumption and utilization, in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, vol. 4, eds. G.A. Kerkut and L.I. Gilbert, pp. 87–163, Pergamon, New York, 1985; Waldbauer, G.P., *Adv. Insect Physiol.*, 5, 229, 1968.

*Notes:* AD, approximate digestibility (also termed absorption efficiency); ECD, efficiency of conversion of digested food to body substance (also termed metabolic efficiency); RCR, relative consumption rate; RGR, relative growth rate (product of RCR × AD × ECD) expressed as the amount of growth attained (mg dry matter) per unit of body weight (mg dry matter) per unit of time.

reproduction. The performance (the extent to which an insect herbivore is able to realize maximum growth and reproduction) is preferentially expressed in rate parameter, and when looking at performance and utilization value, large differences appear to exist between different feeding guilds, such a mandibulate feeders of herbs and forbs versus woody plants or mandibulate versus haustellate (piercing–sucking) species (Table 29.1). Estimates of crop production removed by foliage-feeding insects typically ranges from 5% to 30% (Mattson and Addy, 1975), and insect outbreaks can reduce net primary productivity by more than 70% in some terrestrial ecosystems (Cyr and Pace, 1993; Agarwal et al., 2006). The loss of productivity to herbivory traditionally has been estimated as the amount of leaf tissue removed (Ohmart et al., 1983; Lowman, 1985). It is estimated that insects (mandibulates) consume in the order of 10% of all annually produced plant biomass, these data varies considerably with vegetation type, time, and locality (Barbosa and Schultz, 1987; Damman, 1993; Coupe and Cahill, 2003). Losses to sap-feeding insect (haustellate mouth parts) are more difficult to measure, but are estimated to be around 5% of the net primary production (Schoonhoven et al.,

**TABLE 29.2**  
**Numbers of Herbivorous Species in Some Insect Order**

| Insect Order | No. of Species (Estimate) | Herbivorous Species |     |
|--------------|---------------------------|---------------------|-----|
|              |                           | No.                 | %   |
| Coleoptera   | 349.000                   | 122.000             | 35  |
| Lepidoptera  | 119.000                   | 119.000             | 100 |
| Diptera      | 119.000                   | 35.700              | 30  |
| Hymenoptera  | 95.000                    | 10.500              | 11  |
| Hemiptera    | 59.000                    | 53.000              | 90  |
| Orthoptera   | 20.000                    | 19.900              | 99  |
| Thysanoptera | 5.000                     | 4.500               | 90  |

*Source:* Schoonhoven, L.M. et al., *Insect–Plant Biology*, 2nd edn., Oxford University Press, Oxford, New York, 2005.

2005). Measuring the intensity of insects herbivory is often difficult and estimate of losses can differ two- to fivefold among the methods used (Coley and Barone, 1996; Schowalter, 2000; Peterson and Higley, 2001). Insects often inflict much more damage in agro-ecosystems than in natural settings (Peterson and Higley, 2001). Conspicuous among herbivores are the Lepidoptera (butterfly and moth), Hemiptera (bugs, leafhoppers, aphids, etc), and Orthoptera (grasshoppers and locust), but large parts of Coleoptera, Hymenoptera, and Diptera (Schoonhoven et al., 2005) (Table 29.2) are also present. Tissue damage usually induces local osmotic stress responses that are often found to be a key component in the response to mechanical wounding (Reymond et al., 2000; Deneckamp and Smeeckens, 2003). In this chapter, we analyze in depth how the insect herbivores find host plants and arrive in a patch, how damaging the plant tissue causes stress, how the plant responds to damage, and the effects of this stress on growth and reproduction of the plant.

## 29.2 PLANT INTERACTIONS WITH INSECT HERBIVORES

### 29.2.1 HOW INSECT HERBIVORES FIND A HOST PLANT

Insects are often said to show programmed behavior and stereotyped, predictable sequence of behavioral act called *reaction chains* (Atkins, 1980) and that more or less distinct behavioral elements follow one another in a fixed order after succession of stimuli, as demonstrated by Zohren et al. (1968). The study shows the behavior of *Delia radicum* (cabbage root fly), where a gravid female fly lands on the cabbage leaf in response to yellow-green wavelengths (500–600 nm). Then she walks along the leaf throughout the latent phase, pausing now and then to groom or to make short flights. At the leaf-blade run phase, she walks continuously, repeatedly along the leaf edge and often changing direction. She assesses the suitability of the plant with taste hairs on her tarsi. Then, she moves on to a midrib of a leaf or a stem, once she contacts the appropriate chemical stimuli, followed quickly by stem run. Next, she moves around the stem base, entering the stem circling phase, keeping her head downward, and then climbing up the stem few centimeters. Afterward, she starts probing the soil with her ovipositor waiting for the chemical stimuli that when perceived, she finally lays her eggs in the soil close to the stem (Zohren et al., 1968). When the outcome of a sensory evaluation is the rejection of a particular plant or plant tissue as food or oviposition site, the insect jumps back to earlier steps in the reaction sequence. In the process of host-plant selection, two main consecutive phases may be distinguished: searching and contact-testing. A standardized host-plant selection sequence can be described as follows (Schoonhoven et al., 2005):

- The insect herbivore has no physical contact with a plant and either rests or moves about randomly, walking or flying.
- It perceives plant-derived cues, optical and/or olfactory.
- It responds to these cues in such a way that the distance between its body and the plant decreases.
- The plant is found (contacted by either touching or climbing, or by landing).
- The plant surface is examined by contact-testing (palpation of surface).
- The plant may be damaged and the content of tissue released by nibbling or test-biting (for biting–chewing species), probing (piercing–sucking species), or puncturing with the ovipositor.
- The plant is accepted (one or more eggs being laid or continued feeding) or is rejected, resulting in the insect's departure.

When it arrives in a patch of potential host plants, it may exhibit repetition of the same sequence with respect to different plant individuals of the same or other species. Once an insect has established contact with a potential host plant, elaborate evaluation behavior ensues, during which the insect uses both mechanosensory and chemosensory stimuli offered by the plant. At the behavioral

level, it has been ample documents that acceptance is determined by the balance between stimulatory and inhibitory compounds (Schoonhoven et al., 2005). The crucial decision to accept or to reject a plant is based not only on sensory information of plant cues but also on the insect's physiological status (satiety, sexual maturity, eggs maturation, etc.; Barton Browne, 1993).

### 29.2.2 DAMAGE TO PLANTS CAUSED BY INSECT HERBIVORES

The herbivorous are distinguishable based on the degree of food-plant specialization in monophagous, oligophagous, and in polyphagous. The first occurs only on one or a few closely related plant species; many lepidopterans, hemipterans, and coleopterans fit into this category; the second feed on a number of plant species, all belonging to the same plant family; the latter category are herbivorous that seem to exercise little choice and accept many plants belonging to different plant families. The classification of an insect species into this three categories are difficult to sustain, and it is often more convenient to distinguish the insect herbivorous only in *specialist* (monophagous and oligophagous) from *generalist* (polyphagous species) (Pashley, 1986; Bryne and Bellows, 1991; Howard et al., 1994).

#### 29.2.2.1 Herbivory in Aboveground and Belowground Plant Tissues

For the first category, the insects may consume every anatomical part of plant and also show specialization with regard to feeding site they occupy on their hosts. Many caterpillars, beetles, and grasshoppers are leaf foragers (folivores), ingesting relatively large chunks of leaf material. These extensive tissue damage caused by these insects activate JA-dependent and JA-independent wound response and herbivore responsive genes (Walling, 2000). Other insects such as hemipterans and homopterans show more specific needs. Plant bugs penetrate epidermal cells and ingest cell contents, aphids suck mainly from the sap flow in phloem sieve elements. Spittlebugs and cicadelline leafhoppers often tap the xylem (Thompson, 1994). Phloem-feeding whitefly and aphid cause small wounds in plant foliage that are perceived as pathogens by the plant's defense system and activate the SA-dependent and JA/ET-dependent signaling pathways (Walling, 2000). Another insects of other order are the leaf-mining insects that live and feed during their larval stage between the upper and lower epidermis of leaf blade and devour the parenchymal tissues. In this category, there are some insects that may excavate different layers of the leaf parenchyma. The *Betula papyrifera* leaves, for example, are infested by two hymenopterous leaf miners: the *Fenusa pusilla* feed on the entire mesophyll, whereas the *Messa nana* larvae feed only on palisade parenchyma (DeClerck and Shorthouse, 1985; Scheirs et al., 2001). The different leaf part taste affects not only the leaf miners but also insect ingesting leaf pieces. The larvae of the caterpillars *Lymantria dispar* and *Catocala* spp. can discriminate between the basal, lateral, and terminal leaflets of their compound-leaved food plant and show dislike to basal leaflets (Gall, 1987). Stems of plant may harbor stem-borers as some species of Lepidoptera, Coleoptera, and Hymenoptera, and the bark of woody plants are often infested by the Coleoptera bark beetles (Scolytidae and others). Wood may be used as food by some Lepidoptera, Coleoptera, and Hymenoptera, which are adapted to these unbalanced diets. Some insect species are specialist feeders on flowers, fruits, or seeds, and others are more specialized to induce the formation of galls in various plant parts (Williams, 1994). The variation in adaptations to certain plant tissue is principally due to nutritional factor. The different plant parts show different dietary value, and the smaller the size of the body of herbivore, the finer is the scale of heterogeneity of the plant tissue it meets. For example, for the Lepidoptera polyphagous *Mamestra configurat*, when larvae feeding on the pods of rape, one of their host plants, remain smaller and show a 30% increase mortality rate compared with conspecific larvae feeding on foliage (Bracken, 1984). Another different example is the larvae of *Dasineura brassicae* that are specialized feeders on the pod of rape and survive only on these plant parts (Åhman, 1985). Nutritional factors are not the only determinant of feeding site specialization, several other physiological and ecological factors must also be involved in insects that live belowground. Recent data have shown that the total biomass

of the life belowground is much vaster than all that we observe aboveground. A considerable subterranean herbivore feeds on plant roots, and intimate interactions between insect and plant are likely to mirror the aboveground relationship (Schoonhoven et al., 2005). Root damage may result in inadequate uptake of water, nutrient, mineral, and thereby reduce the growth of aboveground plant part and cause severe yield losses (Maron, 2001). The subterranean herbivory root feeders may also affect their aboveground counterpart and vice versa via changes in their hosts' chemistry or physiology (Van der Putten et al., 2001). For example, the infested root by rice water weevils (*Lissorhoptrus oryzophilus*) markedly reduce the growth rate of fall armyworms (*Spodoptera frugiperda*) feeding on the leaves of the attacked plants, and, reciprocally, severe defoliation by the *S. frugiperda* had a negative effect on performance of rice water weevils (Tindall and Stout, 2001). Herbivores root damage may also affect indirect plant defense in cotton plants (*Gossypium herbaceum*) infested by root feeding wireworms (*Agriotes lineatus*) which increase their extrafloral nectar production in comparison to control plant with their roots intact. Extrafloral nectar production allows plant to recruit predator, which in turn protect the plant against aboveground insect herbivores (Wäckers and Bezemer, 2003). Crop production removed by foliage-feeding insects typically ranges from 5% to 30% (Mattson and Addy, 1975), with maximum to 70% net primary productivity in peculiar ecosystems (Cyr and Pace, 1993; Agarwal et al., 2006). It is widely believed that herbivore–plant interactions and plant defenses vary with latitude. Herbivory is suggested as being more intense and plant defenses are better developed at lower latitudes (Dobzhansky, 1950; MacArthur, 1969; Lowman, 1985; Coley and Aide, 1991; Coley and Barone, 1996; Grime, 2001; Pennings and Silliman, 2005). An increasing foliar damage trend on *Betula pubescens* and *B. pendula* along decreasing latitude was detected in Fennoscandia, while no geographical or climatic pattern was detected on *B. pendula* in its more southern distribution region (e.g., Central Europe) (Kozlov, 2008). Several studies that have estimated rates of folivory have often used quite different methods from one region to another, complicating the comparison (Pennings and Silliman, 2005). This problem appeared to be solved using a closely standardized methodology to compare folivory (not including sap suckers) in selected temperate forest species along a latitudinal gradient using an extensive network of sites (Adams and Zhang, 2009).

### 29.2.2.2 Plant Compensation to Insect Herbivores Damage

Plants are equipped with various mechanisms to reduce the deleterious effects of herbivory. Structurally, plants are modular organisms, which consists of repetitive multicellular units, each with its own meristem and none of these units is vital for the plant as a whole. This particular modularity reduces the adverse effects of herbivory considerably and allows for easy recovery from tissue removal.

How a plant will be affected by herbivory is influenced by variation in environmental conditions (Maschinski and Whitham, 1989); genetic variation in plant response to environmental stress (Rosenthal and Kotanen, 1994; Paige, 1999); phenotypic plasticity (Alward and Joern, 1993); developmental plant stage (Chiarello and Gulmon, 1991; Coley and Barone, 1996; Mercader and Isaacs, 2003; Pedigo and Rice, 2006); the timing (Higley, 1992; Marquis, 1992; Mercader and Isaacs, 2003) or pattern (Meyer, 1998) of herbivory, and type of herbivory (Welter, 1989; Meyer and Whitlow, 1992; Delaney and Macedo, 2001); bottom or up defoliation (Damascos, et al., 2005); plant anatomical characteristic, mode of plant reproduction, stored reserves; and availability of water, nutrient, and light (Whitham et al., 1991; Delaney and Macedo, 2001; Utsumi et al., 2009). These factors influence the effectiveness of plant compensatory responses after herbivory (Nowak and Caldwell, 1984; Maschinski and Whitman, 1989; Herms and Mattson, 1992; Trumble et al., 1993; Rosenthal and Kotanen, 1994). The recovery from herbivory results from the presence of meristem (often dormant) and the ability to redirect resources as nutrient and photosynthetic products to regrowing tissue (Haukioja, 1991).

Under good resource conditions, plants can partially or wholly compensate or overcompensate (production of more biomass than has been lost to herbivory) for losses in feeding (Belsky, 1986).

**TABLE 29.3**  
**Environmental Factors and Plant Traits Involved**  
**in Plant’s Compensatory Response to Herbivory**

| Undercompensation                  | Equal or Overcompensations         |
|------------------------------------|------------------------------------|
| Herbivory late in season           | Herbivory early in season          |
| Low water, nutrients, and/or light | Low water, nutrients, and/or light |
| High competition                   | Low competition                    |
| Meristem limitation                | No meristem limitation             |
| Slow growth                        | Fast growth                        |
| Nonintegrated plant modules        | Integrated plant modules           |
| Woody perennials                   | Annuals and biannuals              |

*Source:* Whitham, T.G. et al., Plant response to herbivory, the continuum from negative to positive and underlying physiological mechanism, in *Plant–Animal Interactions. Evolutionary Ecology in Tropical and Temperate Regions*, ed. P.W. Price, T.M. Lewinsohn, G.W. Fernandes, and W.W. Benson, pp. 227–256, Wiley, New York, 1991.

In fact, Huttunen and colleagues (2007) show that climatic change will have a positive impact on the compensatory ability of defoliated plants when these grow in fertile soil.

Herbivore-induced modifications in plants often have negative impact on the survival and reproduction of insect herbivores because of decrease of morphological resistance (Masters and Brown, 1992; Inbar et al., 1995; Denno et al., 2000; Tindall and Stout, 2001; Wise and Weinberg, 2002; Denno and Kaplan, 2007).

It is difficult in the present state of the art to make generalization regarding the ecological importance of compensatory responses because of many factors involved (Table 29.3).

The compensatory regrowth response can have positive effects on abundance and performance of herbivorous insects via increasing food quality and/or quantity (Danell and Huss-Danell, 1985; Damman, 1989; Strauss, 1991; Martinsen, et al., 1998; Nakamura, et al., 2003). Herbivore-induced plant response also indirectly affects predator abundance and/or predation pressure through bottom-up trophic cascade (Masters et al., 2001; Bailey and Whitham 2003; Nakamura et al., 2005, 2006). Several authors have argued that increased abundance or species richness at lower trophic levels may result in increased abundance or species richness at higher trophic levels (Hunter and Price, 1992; Abrams, 1995; Siemann, 1998). Hence, the plant regrowth response following herbivory may have a subsequent influence on abundance and species richness of predaceous arthropods by altering both species composition and abundance of prey herbivores (Utsumi et al., 2009).

There are differences, moreover, between monocot and dicot plants with respect to optimal conditions for overcompensation. The difference is caused, probably, by a difference in meristem location in the two groups of plants, which entails important physiological consequences. Monocot herbs, in fact, grew more after herbivory in high resource conditions, whereas recovery from herbivory in dicot herbs and woody plants was significantly better in low resource conditions (Hawkes and Sullivan, 2001). Compensation responses to insect that do not destroy the photosynthetic machinery, such as phloem sap-feeding species, are more difficult to measure.

**29.3 PLANT RESPONSES TO INSECT HERBIVORES ATTACK**

Here, we review the latest information on the plant immunity to herbivorous insects based on biochemical and molecular research advances. Insect herbivores use various feeding strategies to get nutrients from their host plants. Being sessile within their local environment and in order to protect

themselves from herbivorous insects, plants have to respond in a rapid and effective way. Therefore, they have evolved an array of sophisticated and highly adaptive responses to herbivory, which include constitutive and inducible defense strategies. Inducible defenses are exhibited in two forms, direct and indirect, and appear to be widespread in the plant kingdom. Inducible direct defenses have been exclusively studied since the 1970s (Green and Ryan, 1972; Farmer and Ryan, 1992; McCloud and Baldwin, 1997; Baldwin and Preston, 1999), whereas the inducible indirect defenses have been studied since the 1980s (Sabelis and van de Baan, 1983; Sabelis and Dicke, 1985; Dicke and Sabelis, 1988; Turlings et al., 1990). Inducible direct defenses include the production of compounds that exert repellent, anti-nutritive, or toxic effects on herbivores as well as physical barriers such as leaf hardness and trichomes that increase plant fitness in the presence of herbivores. Plants respond to insect attack by producing toxins and defensive proteins that target physiological processes in the insect instead of acting as passive victims in these interactions. Major known defense chemicals comprise plant secondary metabolites, protease inhibitors, lectins, and amino acid deaminases and oxidases. Indirect defenses include plant volatiles organic compounds (VOCs) and nectar rewards. Both are induced by insects and play a role in attracting natural enemies of the herbivore (Kessler and Baldwin, 2004). Moreover, herbivorous insects trigger induced defenses both locally and systematically through signaling pathways involving jasmonate, ethylene (ET), and salicylates. The plant immunity to herbivore insects is highly dynamic and is initiated by the recognition of insect oral secretions and signals from injured plant cells. These initial cues are transmitted within the plant by signal transduction pathways that include calcium ion fluxes, phosphorylation cascades, and, in particular, the jasmonate pathway, which plays a central and conserved role in promoting resistance to a broad spectrum of insects (Howe and Jander, 2008). The joint effects of plant direct and indirect defense provide a robust resistance to a broad spectrum of insect herbivores in natural ecosystems (Gatehouse, 2002; Kessler et al., 2004). In addition to induced defensive traits, plants can minimize the fitness consequences of tissue loss by activating physiological processes, such as sequestration of sugars in belowground tissues, which allow the plant to tolerate herbivory better (Schwachtje et al., 2006).

### 29.3.1 EARLY EVENTS IN PLANT–INSECT INTERACTIONS

Plants that are swiftly and accurately able to recognize, decipher the incoming signal, and effectively respond to a wide array of attacking insects are the ones with greater success to withstand insect stress. These events that take place the first seconds to minutes are responsible for recognition and triggering of signal transduction pathways, but they are still poorly understood. They start from damage-induced ion imbalances, variations in membrane potentials, calcium flux, production of reactive oxygen species (ROS), and mitogen-activated protein kinases (MAPK) activities. Additionally, insect elicitors have been identified by many studies to allow plants to differentiate between herbivory and mechanical wounding. Moreover, there is evidence that *R* genes are involved in the control of host plant resistance to insect attack.

Although all herbivory results in plant tissue damage, tissue disruption per se is not always a reliable indicator of insect attack. Therefore, to avoid wasting defensive resources, plants must differentiate insect feeding and simple mechanical damage, such as that caused by hail or wind in natural settings. Some responses, including the up-regulation of genes required for cell repair and response to osmotic stress, would likely occur as a result of either herbivory or mechanical wounding. However, the production of toxic secondary metabolites and other defensive responses would presumably benefit only herbivore-challenged plants (Howe and Jander, 2008).

Plants may differentiate mechanical wounding from herbivory through the recognition of compounds in insect oral secretion as well as through the use of as yet unknown mechanisms that gauge the quantity and quality of tissue damage (Howe and Jander, 2008).

Oral secretions from herbivorous insects elicit volatile release in their host plants. The emission of volatiles is one of the well-studied plant defense responses to insect attack. Numerous plant–insect

interaction studies have shown that insect feeding or application of oral secretions to wound sites elicits a different or more intense volatile response than mechanical damage alone (Turlings et al., 1990; De Moraes et al., 2001; Arimura et al., 2004).

However, it is not the case for aphids, as aphids-derived elicitors are yet to be identified. A study by Miles (1999) showed that aphid salivary enzymes such as peroxidases and pectinase may be elicitors of plant defense responses, yet this hypothesis needs to be further tested. Genetic proof coming from various monocot and dicot crop species supports the idea that *R* gene products mediate resistance to phloem-feeding insects (Smith and Boyko, 2007). Two plant NBS-LRR (nucleotide binding site–leucine rich repeat) proteins that contribute to the recognition of hemipteran herbivores have been isolated: the tomato *Mi-1* gene and the melon *vat* gene. The former provides resistance to some isolates of *Macrosiphum euphorbiae* (potato aphid) and *Bemisia tabaci* (silverleaf whitefly), although not to *Myzus persicae* (green peach aphid) (Rossi et al., 1998; Nombela et al., 2003). The latter confers increased resistance to both *Aphis gossypii* (cotton aphid) and the transmission of plant viruses by this aphid species (Dogimont, 2007). By analogy to plant defense against pathogens, these findings suggest a gene-for-gene interaction between the plant and the aphid. However, the presumed avirulence proteins in aphid saliva have not yet been identified (Howe and Jander, 2008).

Furthermore,  $\text{Ca}^{2+}$  has been implicated as a second messenger in many plant signaling pathways, including responses to herbivory (Maffei et al., 2007). Experiments involving  $\text{Ca}^{2+}$  chelator treatment in *Phaseolus lunatus* (lima bean) resulted in the prevention of defense gene induction in response to feeding by *Tetranychus urticae* (two-spotted spider mite) and volatiles from mite-infested neighboring plants (Arimura et al., 2000).

Despite the fact that MAPK pathway leading to insect resistance has not been identified yet, evidence that such pathway is involved in some plant–insect interactions is shown. *Mi-1* mediated resistance was attenuated when the expression of certain MAPKs and MAPK kinases was reduced by VIGS (virus-induced gene silencing) in tomato (*Solanum lycopersicum*; Li et al., 2006).

### 29.3.2 PLANT DEFENSE SIGNALING PATHWAYS

Salicylic acid (SA), jasmonic acid (JA), and ET are recognized as key players in the regulation of defense responses to insect herbivores that inflict various types of tissue damage in the plant (Howe, 2004; Lorenzo et al., 2004; Pozo et al., 2004; Grant and Lamb, 2006; Van Loon et al., 2006; Harfouche et al., 2006; Von Dahl and Baldwin, 2007). Other plant hormones, including abscisic acid (ABA; Mauch-Mani and Mauch, 2005), brassinosteroids (Nakashita et al., 2003), and auxin (Navarro et al., 2006; Wang et al., 2007), have also been involved in plant defense to insect herbivores, yet their significance needs further studies. By means of synergistic and antagonistic interactions (cross talk), which depend on the herbivorous insect and the attacked plant, a fine-tuned response is achieved that regulates gene expression and, thus, controls the production of protective metabolites (Maffei et al., 2007). Silencing of the octadecanoid pathway in *N. attenuata* resulted in plants that were more vulnerable to insect attack, demonstrating the importance of this pathway for plant defense against insects (Kessler et al., 2004). What is more, the level of SA was raised only moderately in corn (*Zea mays*) (Schmelz et al., 2003) and Arabidopsis (Stotz et al., 2002; Traw et al., 2003) after insect attack, knowing that microbial infections generally caused much higher levels of SA than insect feeding. Also, increase in ET production is another early and active response of plants to insect attack. ET synergizes volatile emission in corn (Ruther and Kleier, 2005) and modulates many other defense responses to insect herbivory (Baldwin et al., 2006; Harfouche et al., 2006). The antagonistic roles played by the JA pathway in affecting plant resistance to chewing and phloem-feeding herbivores in monocotyledonous plants reported by Zhou et al. (2009) question the general assumption that JA-dependent defenses enhance plant resistance against both chewing and phloem-feeding herbivores. The silencing *OsHI-LOX* (a chloroplast-localized type 2 13-lipoxygenase gene of rice) makes rice more susceptible to chewing herbivores, but enhances resistance to a phloem feeder (Zhou et al., 2009).



### 29.3.3 DIRECT DEFENSE RESPONSES

In order to protect themselves against insect attack, plants are equipped with an array of defense mechanisms, one of which is the direct defense. Direct plant defenses can be generally divided into two categories: antinutrition and toxicity. It is likely that all plants exhibit constitutive or induced accumulation of toxic secondary metabolites as part of their defense against herbivory (Howe and Jander, 2008). Research on plant defenses to insect pests using numerous plant species has revealed a large variety of small molecules that have toxic and antinutritional effects. Terpenoids that are the most metabolically diverse class of plant secondary metabolites, with more than 40,000 known structures, play a role in plant defense (Aharoni et al., 2005). Also, alkaloids such as nicotine, caffeine, morphine, cocaine, and strychnine are likely evolved as direct defense against insect pests. Tanins, saponins, glucosinolates, and furaocoumarins are other well-studied classes of plant secondary metabolites that exhibit defensive properties to insect attack.

Advances in plant molecular biology research have led to the identification of defensive toxin biosynthetic pathways. Genes encoding all five enzymes involved in the biosynthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), a toxin found in maize, wheat, and other Gramineae have been discovered (Frey et al., 1997). In addition, most of the genes required for the production of glucosinolates, a diverse class of metabolites found in the model plant *Arabidopsis thaliana* and other Cruciferae, have been identified (Halkier and Gershenzon, 2006). Using this information and to better understand how it can be applied to improve plant immunity to insect pests, Tattersal et al., (2001) have engineered *A. thaliana* with three enzymes from grain sorghum (*Sorghum bicolor*) to produce the cyanogenic glycoside dhuririn, thereby enhancing resistance to yellow-striped flea beetle (*Phyllotreta nemorum*). Many defensive compounds are potentially toxic to the plants that produce them. Therefore, the storage of relatively benign precursors that are activated by herbivory is a recurring theme in plant biology. For instance, all three of the defensive systems mentioned in the above paragraph include compounds that are sequestered in plants, but not activated until the onset of herbivory. DIBOA is stored as inactive DIBOA-glucoside, glucosinolates are enzymatically activated to produce toxic breakdown products, and the respiratory inhibitor hydrogen cyanide is released from cyanogenic glycosides during herbivory (Howe and Jander, 2008). Two aspects should be clarified here, one pertains to the synergistic effects in defense against herbivory that may be provided by the complex mixture of toxins found in many plants, and the other to the metabolic diversity in toxin production by individual plants that can also provide defense against multiple insects with different feeding styles and resistance mechanisms. Hummelbrunner and Isman (2001) demonstrated that combining two monoterpenoids almost increased the toxicity 10 times more to tobacco cutworm (*Spodoptera litura*) than would have been predicted from a simple additive effect. Results of research experiments involving four insect herbivores demonstrated that tryptophan-derived indole and methionine-derived aliphatic glucosinolates have differing effects on Hemiptera and Lepidoptera (Mewis et al., 2005). What's more, a better defense against *M. persicae* has been gained by Indole glucosinolates, which break down in the absence of the activating enzyme myrosinase (Barth and Jander, 2006) than do the more stable aliphatic glucosinolates (Kim and Jander, 2007).

Besides, plants challenged with insect express defensive proteins that exert direct effects on the attackers. The plant's insect defense protein arsenal includes proteinase inhibitors (PIs), cysteine proteases, lectins, chitinases, polyphenol oxidases (PPOs), lipoxigenases (LOXs), arginases, and threonine deaminase (TD), the discovery of which has been eased by proteomic and functional genomics analyses. PIs play an active role in the active defense response by impairing various mechanistic classes of digestive proteases in the insect midgut (Green and Ryan, 1972; Ryan, 1990). PIs inhibit insect gut proteases, which results in amino acid deficiencies that negatively affect the insect growth and development (Zavala et al., 2004; Lison et al., 2006). However, PIs defense efficacy is often foiled by the insect's adaptive ability to express digestive proteases that are insensitive to the host plant complement of PIs or that inactivate PIs (Jongsma et al., 1995; Giri et al., 1998; Rivard et al., 2004;

Bayes et al., 2005). This evolutionary arms race between two distinct defense mechanisms may be reflected by the diversity and rapid evolution of certain PI gene families (Talyzina and Ingvarsson, 2006). Members of the cysteine protease family of enzymes play another role in defending the plant against insect attack by disrupting the chitin-rich peritrophic membrane that protects the gut epithelium (Konno et al., 2004; Mohan et al., 2006). Besides, plant lectins and chitinases may also target carbohydrate-containing components of the insect gut (Peumans and Vandamme, 1995; Lawrence and Novak, 2006). What's more, oxidative enzymes such as PPO and LOX covalently modify dietary protein through the production of reactive o-quinones and lipid peroxides, respectively (Felton et al., 1994; Constabel et al., 1995; Wang and Constabel, 2004). Based on the premise that defensive proteins are relatively resistant to gut proteases and, as a consequence, are highly enriched during passage of the food bolus through the insect (Howe and Jander, 2008), proteomic analysis of gut content and feces (frass) of insect herbivores in tomato-reared *Manduca sexta* (tobacco hornworm) larvae has led to the identification of isoforms of arginase and TD, which degrade the essential amino acids arginine and threonine, respectively, in the lepidopteran midgut (Chen et al., 2005).

### 29.3.4 CHEMICAL COMMUNICATION DURING HERBIVORY

The emission of volatiles is another important plant response affecting insect attackers either directly and indirectly. In response to herbivory, they can provide a direct defensive benefit by deterring further conspecific oviposition (De Moraes et al., 2001) or an indirect benefit by attracting predators (Kessler and Baldwin, 2001). This phenomenon is well studied in many plant species. The specificity of this interaction has revealed with the expression of a *Z. mays* herbivore-induced terpene synthase (TPS10), that forms (*E*)- $\beta$ -farnesene, (*E*)- $\alpha$ -bergamotene, and other sesquiterpenes, in *A. thaliana* (Schnee et al., 2006). Females of the parasitoid *Cotesia marginiventris* were subsequently attracted to TPS10-producing *A. thaliana* by associating this odor with their prey, *Spodoptera littoralis* (Egyptian cotton worm). Due to the fact that wild-type *A. thaliana* does not produce significant amounts of volatile terpenes, this experiment shows that a single herbivore-induced gene from *Z. mays* is sufficient to elicit this indirect defense. More work with *Z. mays* demonstrates that the emission of volatiles provides an indirect defense against underground herbivory. Challenged by western corn rootworm (*Diabrotica virgifera*) larvae, maize roots release (*E*)- $\beta$ -caryophyllene, which attracts *Heterorhabditis megidis* nematodes that feed on the beetle larvae (Rasmann et al., 2005). Treatment of nonproducing plants with (*E*)- $\beta$ -caryophyllene attracted *H. megidis* and resulted in herbivory reduction.

Additionally, volatiles emitted by insect damage communicate at inter- and intra-plant level by providing a signal that allows nearby plants to get ready for an imminent insect attack occurs. This process results in a rapid and robust response to subsequent herbivory and it is called priming (Arimura et al., 2000; Karban et al., 2000; Engelberth et al., 2004). Some of the good examples of inter-plant signaling via endogenous volatiles emission are summarized here. *Nicotiana attenuata* planted adjacent to clipped *Artemisia tridentata* (sagebrush) received a blend of VOCs that affected gene expression and caused more rapid induction of PI production upon subsequent feeding by *M. sexta* (Kessler et al., 2004). Likewise, green leafy volatiles (primarily degradation products of linoleic and linolenic acids) released by *Z. mays* primed plants in close proximity to respond more robustly following mechanical damage and application of caterpillar oral secretions (Engelberth et al., 2004). At first glance, it would appear that eavesdropping on volatile signals should provide a defensive benefit only to the receiving plant. However, in a tree or other large plant, volatiles transferred between branches or leaves of the same individual would potentially allow faster communication of imminent threats than would phloem-mediated propagation of a systemic signal (Howe and Jander, 2008). Such intra-plant chemical-mediated priming has been demonstrated in field experiments, where clipped *A. tridentata* showed that defense priming depends on the movement of an airborne signal between damaged and undamaged branches (Karbon et al., 2006).

## 29.4 DETRIMENTAL EFFECTS OF INSECT HERBIVORES TO PLANTS

### 29.4.1 EFFECTS ON PHOTOSYNTHESIS

Insect infestation and damage are one of the most important crop stresses. Insect herbivore damage is assessed by surveying the amount of tissue removed from foliage. This approach, however, assumes that the remaining leaf tissue functions normally. Plants can replace leaf area loss by herbivores through new leaf production but change the photosynthetic rate of the remaining leaves (Chabot and Hicks, 1982) directly and indirectly, as efficiently showed by Nabity and colleague (2009) who discussed how photosynthesis is affected by arthropod herbivores, and the importance of indirect suppression of the surviving mechanisms compared to the loss of photosynthetic capacity from reduced leaf area (Núñez-Farfán et al., 2007). Application of thermal and fluorescent imaging shows that surviving tissue is adversely affected. Photosynthesis is one aspect of primary physiology common in vast majority of higher plants, and leaf chlorophyll fluorescence is increasingly being used as a sensitive tool to assay for the degree of stress that a plant experiences from chronic or acute form of stress (Roháček, 2002). In the wider sense, photosynthesis is central to the performance of autotrophic plants, not in isolation or unique but combined with the processes determining growth and development as part of the whole organism's function and reproductive performance and survival.

Photosynthetic organisms, therefore, have developed many varied mechanisms to avoid or minimize imbalance and to maintain homeostasis. All mechanisms are based ultimately on gene expression: adaptation to adverse conditions requires changes in expression to alter the amounts and activities of system components to maintain or readjust photosynthetic efficiency under adverse conditions and to counteract abiotic and biotic factors (Lawlor, 2009). Thus, from an ecological (and crop production) perspective, ignoring indirect suppression of photosynthesis by arthropods may underestimate its importance (Peterson and Higley, 1993, 2001; Neves et al., 2006; Lawlor, 2009). These "indirect" effects on photosynthesis may be considerably greater than the direct removal of leaf area (Welter, 1989; Zangler et al., 2002; Aldea et al., 2005). Since 1989, Welter examined an extensive body of literature across multiple guilds and found over 50% of all plant–insect interactions resulted in a loss of photosynthetic capacity. Defoliation generally increases photosynthesis, whereas specialized cell-content feeding decreases photosynthesis. These results were partially revised in a recent literature. Feeding on specialized tissues typically reduces photosynthesis, regardless of whether the infested component is the phloem or xylem (Haile et al., 1999; Macedo et al., 2003a,b; Heng-Moss et al., 2006), the stem (Macedo et al., 2005), or general leaf fluids (Haile and Higley, 2003). There are some evidence indicating that increased photosynthesis occurs in the presence of phloem feeding, particularly when the annual photosynthesis rate is estimated (Dungan et al., 2007). The contrast between the results of Welter following the literature where, defoliation injury often does not alter photosynthetic capacity, within plant families (e.g., legumes and some species of *Asclepiadaceae*) or between hardwoods and crops (Peterson et al., 1992, 1996, 2004; Delaney, 2008); however, there are examples where defoliation reduced (Delaney and Higley, 2006; Retuerto et al., 2006) or increased photosynthesis (Coley and Barone, 1996; Aldea et al., 2005; Dungan et al., 2007; Turnbull et al., 2007).

Insect herbivory, whether defoliation or by feeding on specific tissues (e.g., phloem or xylem), triggers a complex and interacting array of molecular and physiological responses in plants. These responses potentially reduce the photosynthetic capacity in remaining leaf tissues to a greater extent than the direct removal of photosynthetic surface area. For example, the removal of only 5% of the area of an individual wild parsnip leaf by caterpillars reduced photosynthesis by 20% in the remaining foliage (Zangler et al., 2002).

#### 29.4.1.1 Direct Reduction of Photosynthetic Capacity

The removal of leaf tissue by herbivores represents a "direct" reduction of photosynthetic capacity (Nabity et al., 2009).

### 29.4.1.2 Indirect Reduction of Photosynthetic Capacity

In addition to directly damaging photosynthetic tissue, herbivores may indirectly affect remaining leaf tissue by diverting resources to defense or disrupt the transport of nutrient and water (Welter, 1989; Sack et al., 2003; Lawlor, 2009). The insect attack to xylem or phloem may alter water transport, stomatal aperture, and sucrose transport and loading, thereby reducing photosynthesis in remaining leaf tissue (Welter, 1989). Severing tissue vasculature alters leaf hydraulics and, subsequently, nutrient or osmotica transport (Aldea et al., 2005; Sack and Holbrook, 2006). If insect feeding is subtle enough to avoid outright cell rupture, modulation of nutrients sequestered by feeding will alter plant osmotica or sink/source relationships (Girousse et al., 2005; Dorchin et al., 2006). These effects also may be mediated by the plant's response. Insect infestation, or even the perception of attack, can induce a myriad of defense-related responses while concomitantly reducing the expression of photosynthesis-related genes (Kessler and Baldwin, 2002).

Where plant defenses are constitutively expressed, the release of biocidal compounds against infesters may damage photosynthetic or homeostatic mechanisms vital for plant function (e.g., Zangler et al., 2002). Indirect effects of herbivory were assigned to four classes: severed vasculature, altered sink demand, defense-related autotoxicity, and defense-induced down-regulation of photosynthesis (Nabity et al., 2009).

#### 29.4.1.2.1 *Alteration of Photosynthesis and Water Balance*

Damage to leaf venation provoked by insect infestation alters leaf hydraulic conductance thereby reducing stomatal conductance and photosynthesis (Nabity et al., 2009). If there are not alternative pathways for water transport, the consequences of damage to venation can persist for weeks after the initial injury and lead to leaf desiccation (Sack and Holbrook, 2006). The foliage damage injury, which severs venation indiscriminately or feeding on specific tissues, may physically obstruct fluid flow with insect mouthparts (stylets) or cell fragments and alter photosynthesis and water balance in remaining leaf tissue (Reddal et al., 2004; Delaney and Higley, 2006). Skeletonizing of soybean leaves by Japanese beetles increased water loss from the cut edges. Damaging the interveinal tissue increased transpiration by 150% for up to 4 days post-injury, and this uncontrolled water loss had no detectable effect on CO<sub>2</sub> exchange, severed vasculature induced for 2 days increase in photosynthetic efficiency in undamaged tissue of damaged leaves (Aldea et al., 2005; Nabity et al., 2009). The effects of defoliation on photosynthesis seem to be less predictable than damage caused by other feeding guilds. In hardwoods, leaf gall and fungal damage consistently reduced photosynthetic efficiency at distances  $\geq 1$  cm from the point of direct damage, whereas defoliation resulted in only highly local reductions ( $< 1$  mm) in photosynthetic efficiency (Aldea et al., 2006).

#### 29.4.1.2.2 *Alteration of Sink Demand*

Plants respond to herbivory with increased CO<sub>2</sub> uptake and the mechanism typically is linked to compensation or an increase in the sink demand within the leaf (Nabity et al., 2009).

Phloem feeding increased whole-canopy photosynthesis in beech trees, perhaps through a reduction in photosynthate buildup; however, the mechanism remains unclear and may be as simple as herbivore preference for hosts with higher rates of photosynthesis (Dungan et al., 2007). Gall formation in red maple, pignut hickory, and black oak reduced photosynthetic efficiency, but increased non-photochemical quenching, indicating a down-regulation of the photosystem II reaction centers in the area around galls (Aldea et al., 2006). A decline in leaf temperature near galls suggests that transpiration was greater and fluid and nutrient transport increased near the point of damage (Macfall et al., 1994). Gall-forming insects, a leaf-mining moth that lives enclosed within leaf tissue of apple trees, instead, reduced carbon assimilation rates by decreasing transpiration (Pincebourd et al., 2006). Defoliation, as well as removal of reproductive and other vegetative sinks, may improve photosynthesis in remaining leaf tissue by increasing carboxylation efficiency and the rate of ribulose-1,5-bisphosphate regeneration (Layne and Flore, 1992; Holman and Oosterhuis, 1999; Thomson et al., 2003; Ozaki et al., 2004; Turnbull et al., 2007).

#### 29.4.1.2.3 Autotoxic Effect of Defensive Compounds on Photosynthesis

Taxa, habitat, and resource availability influence differently the plants defenses (Fine et al., 2006), and many chemical defenses are known for both model plant systems and across less-studied taxa (Coley and Barone, 1996; Berenbaum and Zangerl, 2008). Plants run the risk of autotoxicity because of the biocidal properties of many secondary compounds. The autotoxic effect of defensive compounds on photosynthesis is highly species-specific (Nabity et al., 2009). The secondary compound may reduce the photosynthesis as reported for the wild parsnip (*Pastinaca sativa*). Wild parsnip contains an arsenal of defense compounds including furanocoumarins, which are photoactivated and biocidal against a variety of organisms (Arnason et al., 1991). Furanocoumarins are contained in oil tubes under positive pressure and bleed profusely from the wounding site (Gog et al., 2005). If herbivore's damage involves these tubes, the release of furanocoumarins will reduce the photosynthetic efficiency and gas exchange at considerable distances from the actual point of insect damage (Zangler et al., 2002; Gog et al., 2005).

#### 29.4.1.2.4 Down-Regulation of Photosynthesis-Related Genes

Photosynthetic proteins are often down-regulated by insect attack. Jasmonates play a central role in regulating plant defense responses to herbivores, as shown in the previous chapter, but while jasmonates induce defenses, they also inhibit growth and photosynthesis (Giri et al., 2006; Zavala and Baldwin, 2006; Yan et al., 2007). Transcriptional analysis of plant-herbivore interactions revealed that photosynthesis-related genes are down-regulated after infestation (Hui et al., 2003; Reymond et al., 2004). Herbivorous insect attack reduces a plant's photosynthetic capacity more than is expected, given the canopy area removed by the herbivore (Zangler et al., 2002), a result that points to herbivore-induced reductions in ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) activase (RCA) and RuBPCase/RCA by gene silencing (Hermesmeier et al., 2001; Hahlbrock et al., 2003; Hui et al., 2003) as a potential explanation for the large decrease in the carbon and nitrate assimilation in *Nicotiana attenuate*, followed by reduction in rate of biomass accumulation that characterizes herbivore-attacked leaves (Giri et al., 2006; Mitra and Baldwin, 2008).

Partial defoliation of individual leaves by herbivores largely increases evapotranspiration via enhanced water loss from cut edges and produces leaf dehydration (Aldea et al., 2005), reduces photosynthesis by causing stomata to close, and also by initiating senescence signaling (Lim et al., 2007). It has been suggested that the slower growth and down-regulation of photosynthetic-related genes by herbivore elicitation may be required to free up resources for defense-related processes (Baldwin, 2001).

### 29.4.2 EFFECTS ON YIELD

The perception that any biotic stress intervening on plants, both cultivated and not, interfere negatively with the yield, proportionally with the growing numbers of pests, is known by far, and constitutes the origin of the basic concepts of biotic stress and yield loss. Even if it would seem an obvious concept without deep knowledge of the physiological mechanisms involved in it, nevertheless, a huge scientific effort was devoted to assess and elucidate the role of biotic stressors on yield loss since the birth of modern agriculture. Actually a lot is known about both pests and plants, but it could be stated that the intimate relationship between biotic stress and yield still remains almost unclear.

#### 29.4.2.1 The Yield Loss

The amount of valuable products obtained from crop plants is commonly referred to as "yield" (Madden, 1983). Its estimation can be quantitative (weight of the production) or qualitative.

If every factor involved in the production is at its optimal level, the highest attainable yield is theoretically obtainable; but natural conditions are very rarely optimal and the actual yields are vastly below this value. Bingham (1967) first discerned between the "potential yield" obtainable by

cultivation when free from hazards such as staying, cold, diseases and pest insects, and the “realized yield” as a result of stresses. Yield loss assessment tries to account for the difference between actual and attainable yield. Thus, the loss is considered as the computable reduction both in quantity and/or quality of the attainable production. The losses owing to pest insects represent only one of a quantity of complex production limitations. Its evaluation and prediction, as accurate as possible, in connection with the estimation of the stressor presence, are also crucial for control decisions to be made in the Integrated Pest Management (IPM) programs (Smith et al., 1984).

#### **29.4.2.2 Yield Losses Assessment**

Crop losses due to insects constitute one of the most significant constraints worldwide to the increase of global productivity and food production (James et al., 1991) even if it is almost impossible to determine precisely the value of losses. The importance of such information and the proper approach for collecting data have been discussed many times (James, 1974; Close et al., 1978; James and Teng, 1979, Teng and Krupa, 1980; Madden, 1983, Teng, 1987, etc.). The yield loss concept strictly depends on the definition of injury. Injury is a stimulus producing an abnormal change in a plant's physiological process. Higley and colleagues (1993) defined several kinds of injuries related to the categories of their physiological impact. These consist of population or stand reduction, leaf-mass reduction, leaf photosynthetic-rate reduction, leaf senescence alteration, light reduction, assimilate removal, water balance disruption, seed or fruit destruction, architecture modification, and phenological disruption.

The type of injury does not solely determine yield loss; it has to be coupled with its importance and extent to correctly evaluate the influence on the yield loss. The relationship between injury and yield can be mathematically described as “damage curve.” The theoretical and empirical basis for the damage curve was first established in 1961 by Tammes. Then, the categorization of specific portions of the curve that are indicative of unique types of response between injury and yield was proposed in 1986 by Pedigo and colleagues who also stated that any real pest loss curve can be represented by a portion or the entire generalized curve. However, the response to injury can be categorized in three main universal circumstances: a tolerant response, in which a certain amount of injuries is tolerated by the crop before its yield starts to decline linearly with the increase of injury; a susceptible response, in which a modest compensation occurs and yield declines proportionally with the increase of injury; and a hypersusceptible response, in which the greatest yield loss occurs at low levels of injury and incremental losses become smaller as injury increases. The main difference between curves is in the response of yield to low levels of injury. Consequently, a precise description of the damage at low levels of injury is crucial to any pest management decisions.

#### **29.4.2.3 Economic Injury Level and Economic Threshold**

These concepts are keystones of the present IPM (Stern et al., 1959; Pedigo and Higley, 1992; Pedigo, 1996; Stejskal, 2002). Originally Stern et al. (1959) defined the economic injury level (EIL) as the lowest population density that will cause enough economic damage to justify the cost of artificial control measures. Then, Southwood and Norton (1973) and Ramirez and Saunders (1999) redefined EIL as the pest density at which the cost of additional control is equivalent to the economic loss prevented by realizing the control measure. The American Phytopathological Society (Nutter et al., 1993) advises changing EIL into the counterpart economic damage level (EDL) because the decision thresholds are based on both injury and price.

The economic threshold (ET) is the critical pest population size in which management action should be taken to prevent reaching EIL (EDL) and is an operational criterion instructing when to apply the control measure.

#### **29.4.2.4 Damage in Natural and Agricultural Environments**

One of the most contradictory observations in nature is that very little or even no clear injuries can be observed in plants grown in natural ecosystems despite the impact of a countless number of herbivorous insect species. Nevertheless, it is estimated that insects wipe out approximately 10%

of the annually produced plant biomass with significant variations according to vegetation type, time, and locality (Barbosa and Schultz, 1987; Coupe and Cahill, 2003); in the United States and Canada, the annual loss of forest trees referable to insects has been evaluated to 14% and 22%, respectively (Haraus and Pimentel, 2002). Even when the damage level would seem quite limited, it can seriously affect the plant's health. For instance, it has been calculated that, as a very general approximation, the 10% loss to insects is of the same magnitude as the energy that plants devote to reproduction. On oak (Crawley, 1985), even modest insect damage can slow down seriously the production of seeds: the number of acorns produced per shoot of trees protected by insecticides was four times higher than untreated ones, demonstrating that even low herbivory impact often has potent effects on seed production.

Clearly, the intensity of insect attack may vary among plant communities and plant species, and our limited knowledge prevents us from making any sound generalization. Insects often inflict much more damages in agro-ecosystems than in natural settings.

Estimates of actual losses in crop production worldwide were published by Cramer (1967) and updated nearly 30 years later by Oerke et al. (1994). Despite intensive use of insecticide, crop losses to insect feeding in the United States amount to 13%, whereas worldwide this percentage reaches 15% or more. About 1000 insect species attack agricultural crops in the United States; this number would grow to about 9000 on a worldwide scale, although less than 5% are considered to be serious pests. Insect pest species are predominantly specialist feeders: 75% of temperate and 80% of tropical lepidopterous pests are monophagous or oligophagous.

Loss data for the eight major food and cash crops, wheat, rice, maize, barley, potatoes, soybean, sugar beet, and cotton, have been recently estimated by Oerke and Dehne (2004). As such, in wheat production, the total actual losses vary from 14% in Northwest Europe to 35% and above in Central Africa, Southeast Asia, Commonwealth of Independent States, and Oceania, and the pests are estimated to be about 9% potential losses; in rice production, the total actual loss rates range from 23% in Oceania to 52% in Central Africa and the total loss potential of pests accounts for 65%–80% of attainable yields; also, in potato cultivation, the actual total losses fluctuate from 24% in Northwest Europe to about 50% in Central Africa and the loss estimates worldwide for pests summarized for 18%.

## 29.5 MOLECULAR APPROACHES TO INSECT RESISTANCE

Alarming, losses to insect herbivory come to an estimated 10%–20% for major crops and are a significant limiting factor to food production (Ferry et al., 2004). In an attempt to increase agricultural productivity to feed a world human population that is increasing at the rate of 1.2% per year, in a sustainable and environmentally friendly way, new insights gained at the plant–insect interface are highly indispensable. Despite the fact that plants and crops overexpressing *Bacillus thuringiensis* (*Bt*) toxins have been successful in protecting agricultural crops against insect pests and in reducing the insecticides applications, there is still a call for developing additional and alternative strategies for breeding insect resistance in plants.

A better understanding of the diversity of plant responses to insect attack, and, in particular, the induced defenses and their regulation, has led us to pay closer attention to alternative strategies to protect plants and crops from insect pests by exploiting the endogenous resistance mechanisms exhibited by plants to most herbivorous insects. Recent transcriptomics studies have demonstrated that JA cascade plays a central role in transcript accumulation in plants exposed to herbivory (see above). In addition, SA, ET, and JA signaling molecules do not work to activate defenses independently by linear cascades, but rather establish complex interactions that determine specific responses (Ferry et al., 2004). More insights into the cross talk between defense signaling pathways that are thought to help the plant decide which defensive strategy to follow, depending on the type of attacker it is encountering, can be exploited in the rational design of transgenic plants with increased insect resistance (Rojo et al., 2003). Experiments attempting to determine SA's relation to ET biosynthesis and

defense gene expression have shown conflicting results. To confront this, Harfouche et al., (2008) developed an *in vitro* model system to investigate how SA affects ET biosynthesis, hydrogen peroxide ( $H_2O_2$ ) production, and endochitinase gene expression in the European chestnut (*Castanea sativa*). Further, the model system developed should facilitate the deciphering of defense signaling pathways and their cross talk in plants and forest tree species (Harfouche et al., 2008).

More insect-defense signaling pathways might remain to be deciphered. For example, the discovery of electrophilic cyclopentenone oxylipins (oxygenated fatty acids; well reviewed by Farmer et al. (2003) provided evidence for another signaling molecule involved in local and systemic responses (Li et al., 2002).

Likewise, the manipulation of VOCs biosynthesis represents another strategy leading to the development of insect-resistant crops by making them more attractive to herbivore natural enemies. Degenhardt et al. (2003) discussed the potential of modifying terpene emission with the aim of making crops more attractive to herbivore natural enemies. Additionally, transgenic potatoes in which production of hydroperoxide lyase (the enzyme involved in green leaf volatile biosynthesis) was reduced were found to support improved aphid performance and fecundity, suggesting toxicity of these volatiles to *M. persicae* (Vancanneyt et al., 2001).

Also, a better understanding of the responses in herbivores to plant defenses (exposure to PIs) is of paramount importance to crop protection if this strategy is to succeed. Continuing efforts to produce transgenic plants resistant to insects are taking place even though some insects have the abilities to adapt to PIs. For example, Rahbé et al. (2003) investigated the effects of cysteine protease inhibitor oryzacystatin (*OC-1*) on different aphid performance of *M. persicae* using *OC-1* expressing transgenic oilseed rape. De Leo et al. (2001) show that the effectiveness of a PI against a given insect pest is related to its expression level in plants, its activity toward the targeted insect proteinases, and the adaptive capacity of the target insect. The ability of pests to adapt to specific PIs is species-specific; for example, expression of a mustard trypsin inhibitor (*MTI-2*) in oilseed rape resulted in high mortality and significantly delayed larval development of diamond-backed moth (*Plutella xylostella*), but not of armyworm (*S. littoralis*).

What's more, the applications of plant endogenous signaling molecules, SA, ET, and JA, should also be a part of a new approach in the IPM in plants. However, intensive research is needed to develop practical elicitor-based applications in pest and disease management (Holopainen et al., 2009).

Likewise, plant volatiles play a decisive role in the plant–insect chemical communication and regulation of insect behaviors. Although the use of plant volatile for monitoring and controlling insect pests, such as simple inexpensive sticky traps, has become standard monitoring tools in recent years, it should be emphasized that the first efforts to apply semiochemicals for crop protection be made with natural plant volatile (Ti and Zhang, 2009).

Last but not least, developing genetically modified plants that use RNA interference (RNAi) to kill the insects that eat them started to demonstrate the promise of using this technology as a means of pest control. Recent experiments show that in some insects, eating double-stranded RNA (dsRNA) is enough to cause gene silencing. Mao et al. (2007) made cotton plants that silence *CYP6AE14* (a cytochrome P450 gene), a gene that allows cotton bollworms (*Helicoverpa armigera*) to process the toxin gossypol, which occurs naturally in cotton. Bollworms that eat the genetically engineered cotton cannot make their toxin-processing proteins, and they die.

The approaches discussed here are an integral component of IPM strategies. In order to breed a robust multimechanistic resistance to insect pests in plants and crops, the increased knowledge gained by plant breeders and biotechnologists is to be exploited.

## 29.6 CONCLUSIONS

Plant–insect interactions research is arguably one of the most multidisciplinary endeavors in plant biology. Using the available tools of molecular and cell biology, functional genomics, transcriptomics, genetics, biochemistry, and physiology, plant scientists are shedding more light on the complexity



and dynamics of plant–insect interactions. This chapter reviews the most up-to-date advances in the research of plant–insect interface. Environmental conditions that decrease growth, reproduction, etc., are often called “stresses,” but it is not the environment that is “stressful.” Environment is neutral: there is no such thing as a stressful environment *per se*. An environmental factor is only stressful if the organism is unable to function effectively (Lawlor, 2009). It is when environmental conditions exceed the ability of individuals in species to achieve optimal performance that the environment becomes a “stressor.” Different anomaly of environmental factors, abiotic such as temperature, water, and light, and biotic such as insect pests or pathogens, may determine crop stress. Under such conditions, by definition, plants photosynthesize, grow, produce, and survive suboptimally, and of course they will not outcompete better-adapted species (Lawlor, 2009). One of the major stress is caused by tissue damage. Tissue damage in plants is associated most often with insect herbivore infestation. Tissue damage usually induces local osmotic stress responses that are often found to be a key component in the response to mechanical wounding (Reymond et al., 2000; Deneckamp and Smeeckens, 2003). In this chapter, we analyzed how the insects herbivores find and arrive in a patch of potential host plants, how much damage is caused to the plant tissue by stress, how the plant responds to damage and the results of this stress on growth and on reproduction plant. The last interesting data are that the process of priming or hardening involves prior exposure to a biotic or an abiotic stress factor making a plant more resistant to future exposure. This feature, in higher plants, indicates some capacity for “memory” (Bruce et al., 2007), which should be better investigated in future scientific work. We hope that this chapter conveys to readers a clear picture of plant–insect interactions and inspires more generations of biologists to pursue its study. Finally, we apologize to all our colleagues whose work could not be reviewed here owing to space limitations.

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# 30 Stress in Plants and Crops Induced by Herbicide-Mediated Alteration in the Population and Activity of Root-Associated Microorganisms

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## 30.1 INTRODUCTION

There has been a great deal of increase in the use of herbicides in the world during the past decades (Altman and Campbell, 1977; Altman and Rovira, 1989; Heydari and Misaghi, 2003; Moorman and Dowler, 1991; Utkhede, 1982). New and more effective herbicides are continuously being developed to replace the old ones. In the course of their development, herbicides are routinely screened for the absence of phytotoxicity to crops. However, little attention has been given to the possibility that they may also be toxic to plant-associated microorganisms such as those that cause disease and those that protect crops against diseases (Heydari and Misaghi, 2003; Weller, 1988). Consequently, most herbicides are biologically active against nontarget microorganisms (Heydari and Misaghi, 2003; Rodriguez-Kabana and Curl, 1980). Some herbicides have been shown to cause changes in populations of some microorganisms, including bacteria in the soil (Atlas et al., 1978; Heydari and Misaghi, 1998a; Moorman and Dowler, 1991) and in the rhizosphere (Heydari and Misaghi, 2003; Mudd et al., 1985). Herbicides are also known to cause changes in the incidence and severity of some plant diseases, possibly by affecting plant pathogens (Ahmad et al., 1995; Altman and

Campbell, 1977; Altman and Rovira, 1989; Mekwatanakarn and Sivasithamparam, 1987; Rovira and McDonald, 1986).

In this chapter, we will discuss the impact of herbicides on soil-residing plant pathogenic microorganisms that cause disease in plants and on root-associated beneficial microorganisms that suppress the activity of plant pathogens (biocontrol agents). We will show that herbicide-mediated changes in the activity of plant pathogens and biocontrol agents may result in increase or decrease in pathogen-induced stress in plants.

We have been interested in the impact of herbicides on the activity of biocontrol-active microorganisms and on the intensity of pathogen-induced stress because (a) herbicide concentration varies drastically in the soil, (b) microorganisms and plants are sensitive to changes in the levels of herbicides, and (c) unlike other soil factors (temperature, moisture, etc.) the impact of herbicides on the activity of biocontrol microorganisms has virtually been neglected by plant protection researchers and specialists.

### 30.2 PLANT PATHOGENS AND THE DISEASES CAUSED BY THEM

Plants, like other organisms, have natural enemies that are capable of reducing their vigor and/or destroying them completely. These enemies are among both prokaryotic and eukaryotic groups, including plants, fungi, bacteria, viruses, nematodes, and a few other microorganisms. These pathogens attack plants both at above-ground and below-ground parts. The damage to plants caused by insects is not traditionally considered a disease problem.

In a typical scenario, a pathogen is disseminated by wind, irrigation, or floodwater and/or by insects and is deposited near a plant. Some pathogens, like nematodes and flagella-equipped bacteria and fungi, are attracted to plant surfaces using chemotactic responses. Others are deposited on plant surfaces passively. The first step in the infection process is penetration of the pathogen into plants through wounds and natural openings such as stomata. Some pathogens are capable of penetrating plant cells actively by dissolving physical barriers by the aid of enzymes they secrete. Penetration into plant tissue often is followed by a rapid development and spread of the pathogen inside plants.

The outcome of plant–pathogen interaction, which determines the success or the failure of the pathogen to cause disease, depends on a number of factors, including virulence of the pathogen, susceptibility of the host plant, and the prevailing environmental factors (Heydari and Misaghi, 2003; Misaghi, 1982). All of the stages in disease development discussed earlier are sensitive to changes in environmental conditions, including temperature, moisture, soil texture, pH, soil atmospheric composition, and the presence of chemicals such as insecticides, fungicides, and herbicides in the soil. Environmental factors affect disease development by modulating the growth and the activity of pathogens, by interfering with plants' ability to defend themselves against pathogen invasion, and by affecting the development and the activity of microorganisms that compete with pathogens for food resources and for sites on/and around plants.

### 30.3 STRESS IN PLANTS INDUCED BY PLANT PATHOGENS

Plants often suffer from mild and acute forms of stress in response to pathogen invasion. The stress is manifested by a variety of symptoms such as wilting as a consequence of water stress, chlorosis mainly due to chlorophyll breakdown, and necrosis as a consequence of the action of pathogen-produced toxins. Water stress can be induced by destruction of root systems, pathogen-induced change in the permeability of root, and/or leaf cells and by blocking of xylem elements. Pathogen-induced stress may also be manifested as abnormal growth and malformation due to hormonal imbalances. Most pathogens rob plants of vital nutrients, creating nutritional stress. Some of these stressful conditions may be relieved when environmental conditions are altered in ways that are no longer conducive to the growth and pathogenesis of pathogens. However, most plants suffering from

acute forms of pathogen-induced stress often fail to recover. Pathogen-induced stress in some cases may be very important and can potentially result in quantitative and qualitative reduction in plant and crop yields.

### 30.4 BIOLOGICAL CONTROL OF PLANT PATHOGENS

Plant diseases are being controlled mainly by the use of chemicals (fungicides, nematicides, bactericides, etc.), and in some cases by cultural practices. Chemical approaches to managing plant diseases have in recent years been the subject of public concern because of harmful effect of chemicals on the environment, effect on nontarget organisms, and possible carcinogenicity of some chemicals (Heydari et al., 2007; Heydari and Misaghi, 2003). Other problems include appearance of new races of pathogens that are resistant to chemicals, a gradual elimination, and phasing out of some pesticides and reluctance of some chemical companies to develop and test new chemicals due to long registration process and escalating cost (Heydari et al., 2007; Heydari and Misaghi, 2003).

A promising new approach to controlling plant diseases is the use of biological agents and/or their products, an approach known as biological control (Cook and Baker, 1983; Heydari et al., 2007). Biocontrol is environmentally safe and in some cases is the only option available for protecting plants against diseases (Deacon and Berry, 1993; Heydari and Misaghi, 2003; Schroth and Hancock, 1981). Biological control employs natural enemies of pests or pathogens to eradicate or control their population. This can involve the introduction of exotic species, or it can be a matter of harnessing whatever form of biological control exists naturally in the ecosystem. The induction of plant resistance using nonpathogenic or incompatible microorganisms is also a form of biological control. A number of beneficial microorganisms including fungal and bacterial antagonists have been used to combat and control plant diseases in recent years. Some of the important diseases that have successfully been controlled by the use of biocontrol-active microorganisms include cotton seedling damping-off (Heydari et al., 2005; Heydari and Misaghi, 1998a,b, 2003; Jahanifar et al., 2008; Janlou et al., 2008; Misaghi et al., 1998; Zaki et al., 1998), cotton verticillium wilt (Naraghi et al., 2004, 2007, 2008), sugar beet seedling damping-off and root rot (Shahraki et al., 2008, 2009), potato bacterial wilt (Shahriari et al., 2005, 2006), citrus bacterial canker (Khodakaramian et al., 2008), hazelnut decline (Gentili et al., 2008), and turfgrass take all (Heydari, 2007).

Unfortunately, the development of biocontrol strategies to combat plant diseases has been painfully slow. Many biocontrol agents perform consistently and efficiently under laboratory conditions but fail to do so in the field (Heydari et al., 2005; Heydari and Misaghi, 2003; Weller, 1988). This is perhaps because environmental conditions in the laboratory are often artificially selected to be conducive to the development and functioning of biocontrol agents (Heydari and Misaghi, 2003; Weller, 1988). Environmental conditions in the field on the other hand are too complex and exert influence on the activity and performance of microbial biocontrol agents as well as disease incidence and disease development. Moreover, bacteria, fungi, and nematodes are highly sensitive to changes in environmental conditions (Forlani et al., 1995; Heydari et al., 2007; Kim and Misaghi, 1996; Macauley and Griffin, 1969; Misaghi et al., 1992). These observations are the basis for the generally accepted view that a better understanding of the impact of soil and environmental factors on biocontrol microorganisms is the prerequisite for transferring plant disease biocontrol strategies from laboratory to the field (Heydari and Misaghi, 2003; Loper et al., 1984; Weller, 1988). Soil environmental factors that can potentially affect the intensity of stress induced by soil-residing pathogens, populations, and biocontrol activity of microbial biocontrol agents in the field including moisture, temperature, pH, texture, organic content, soil atmospheric composition, and the presence of agricultural chemicals in the soil such as herbicides (Agrios, 1988; Heydari and Misaghi, 1998b; Heydari et al., 1997; Misaghi, 1982).

In this chapter, we will present evidences that preplant herbicides that are being used heavily on variety of crops throughout the world may change the intensity of pathogen-induced stress

(in the presence and absence of biocontrol agents) by influencing plants, pathogens and/or microbial biocontrol agents. We have previously provided evidence that oxygen and carbon dioxide concentrations affect pathogen-induced stress levels by affecting the activity of biocontrol agents (Kim and Misaghi, 1996).

### 30.5 IMPACT OF HERBICIDES ON PATHOGEN-INDUCED STRESS IN PLANTS

Herbicides are known to alter the incidence and severity of pathogen-induced stress in plants (Altman and Campbell, 1977; Altman and Rovira, 1989; El-Khadem et al., 1979, 1984; El-Khadem and Papavizas, 1984; Heydari et al., 2007; Heydari and Misaghi, 2003; Moustafa-Mahmoud et al., 1993; Miller et al., 1979; Neubauer and Avizohar-Hershenson, 1973; Pinckard and Standifer, 1966; Rovira and McDonald, 1986; Wilcox, 1996). Altman and Campbell (1977) showed that the incidence of sugar beet seedling death caused by *Rhizoctonia solani* increased significantly after application of cyclamate to the field soil. Rovira and McDonald (1986) showed that the application of chlorsulfuron to the field soil caused a significant increase in the level of disease stress to wheat and barley caused by *R. solani* and *Gaeumannomyces graminis* var *tritici*, respectively. The severity of stress in cereals due to infection by *Heterodera avenae* increased after application of trifluralin to the field soil (Altman and Rovira, 1989). Disease stress in wheat caused by *Gaeumannomyces graminis* var *tritici* infection (Rovira and McDonald, 1986) and in sugar beet caused by *R. solani* infection (Altman and Campbell, 1977) increased after application of trifluralin, chlorsulfuron and cyclamate, respectively. Application of trifluralin and dinitramine to the field soil also increased *R. solani*-induced stress in cotton seedlings (El-Khadem and Papavizas, 1984; Filippi et al., 1987). In contrast, the severity of *Fusarium oxysporum vasinfectum*-induced stress in cotton plants decreased following application of trifluralin, dinitramine, fluometuron, diuron, dalapon, and prometryn to the field soil, while the incidence of *R. solani*-induced cotton seedling death was not significantly affected by these herbicides (El-Khadem et al., 1984).

We have studied the impact of three preplant herbicides, pendimethalin, prometryn, and trifluralin on *R. solani*-induced stress in cotton seedlings in the microcosm and in the field in Arizona at two locations (Safford and Tucson). Plants attacked by this pathogen may be killed prior to or after emergence. In our microcosm experiments, preemergence and postemergence seedling death were increased in soils treated with two of the three herbicides. In preemergence seedling, damping-off experiment in the microcosm, the stand count (number of emerged seedlings) in the soil treated with prometryn was significantly ( $P < 0.05$ ) dropped by 21%, 36% and 67% relative to the control 1, 2, and 3 weeks after sowing, respectively (Heydari and Misaghi, 1998). The stand count in the soil treated with pendimethalin and trifluralin were not significantly ( $P > 0.05$ ) different from that in the control. In postemergence seedling death experiment in the microcosm, the disease incidence in soils treated with pendimethalin and prometryn increased significantly ( $P < 0.05$ ) by 64%, 60%, 50% and by 64%, 59%, 57%, relative to the control 1, 2, and 3 weeks after inoculation, respectively. The disease incidence in the soil treated with trifluralin was not significantly ( $P > 0.05$ ) different from the control (Heydari and Misaghi, 1998).

Results of field studies corresponded with those of microcosm experiments (Tables 30.1 and 30.2). The stand count in plots treated with pendimethalin and prometryn at Safford significantly ( $P < 0.05$ ) decreased by 30%, 28%; 39%, 30%; and 47%, 39%, for 15, 25, and 50 days after sowing, respectively (Table 30.1). The difference in the amount of disease in plots treated with trifluralin and in nontreated plots at Safford was not significant ( $P > 0.05$ ), relative to the control. The stand count in plots treated with prometryn at Tucson significantly ( $P < 0.05$ ) decreased by, 41%, 49% and 54% relative to the control, 15, 25 and 50 days after sowing, respectively (Table 30.2). Pendimethalin and trifluralin did not cause significant changes in the stand count at Tucson (Table 30.2).

**TABLE 30.1**  
**Plant Stand for Soils Treated with Each Test**  
**Herbicide and Infested with *Rhizoctonia solani***  
**Inoculum for the Safford Field Experiment**

| Treatment                        | Time (Days after Sowing) |           |           |
|----------------------------------|--------------------------|-----------|-----------|
|                                  | 15                       | 25        | 50        |
| <i>R. solani</i> only            | 166(14) a                | 126(12) a | 105(12) a |
| <i>R. solani</i> + pendimethalin | 117(19) b                | 77(19) b  | 56(11) b  |
| <i>R. solani</i> + prometryn     | 121(18) b                | 88(16) b  | 64(15) b  |
| <i>R. solani</i> + trifluralin   | 163(16) a                | 132(13) a | 112(8) a  |

*Notes:* Stand is represented as mean (the average number of emerged seedlings in one plot or one replicate sown with 400 seeds). Each mean is an average of four values. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

**TABLE 30.2**  
**Plant Stand for Soils Treated with Each Test**  
**Herbicide and Infested with *Rhizoctonia solani***  
**Inoculum for the Tucson Field Experiment**

| Treatment                        | Time (Days after Sowing) |           |           |
|----------------------------------|--------------------------|-----------|-----------|
|                                  | 15                       | 25        | 50        |
| <i>R. solani</i> only            | 101(13) a                | 87(11) a  | 79(18) a  |
| <i>R. solani</i> + pendimethalin | 91(56) a                 | 75(43) ab | 55(12) ab |
| <i>R. solani</i> + prometryn     | 60(28) b                 | 44(21) b  | 36(15) b  |
| <i>R. solani</i> + trifluralin   | 104(24) a                | 97(21) a  | 70(11) a  |

*Notes:* Stand is represented as mean (the average number of emerged seedlings in one plot or one replicate sown with 400 seeds). Each mean is an average of four values. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

The reported impact of herbicides on the intensity of pathogen-induced stress has not been always the same in different studies. For example, prometryn, which increased cotton seedling mortality in our studies, did not do so in a previous study (El-Khadem et al., 1984). Moreover, trifluralin that has been reported to increase *R. solani*-induced cotton seedling death (Moustafa-Mahmoud et al., 1993) did not affect the disease in our study. Differential responses may be due to the differences in soil moisture, soil temperature, herbicide concentration, races of pathogens, plant varieties, the composition of rhizosphere microflora, and the rate of herbicide inactivation in various experiments. The development of tolerance to herbicides by pathogens as a result of a long-term herbicide use may also be a contributing factor.

30.6 EFFECT OF HERBICIDES ON POPULATION OF BIOCONTROL BACTERIA IN THE RHIZOSPHERE

To assess the impact of herbicides on the activity of biocontrol bacteria, it is necessary to examine the impact of herbicides on rhizosphere populations of biocontrol bacteria. This is because the ability of these bacteria to develop in the rhizosphere of target plants is a prerequisite for their biocontrol activity (Filippi et al., 1995). Any soil factors which can potentially interfere with the ability of biocontrol bacteria to develop in the rhizosphere is expected to affect their biocontrol activity as well. However, despite its importance, as far as we have been able to determine, except for our study (to be discussed below), the impact of herbicides on population of biocontrol bacteria in the rhizosphere has not been studied.

We studied the potential impact of three widely used herbicides, pendimethalin, prometryn, and trifluralin, on populations of five plant disease-suppressing bacterial isolates (three isolates of *Pseudomonas fluorescens* and two isolates of *Burkholderia cepacia*) in the rhizosphere of cotton seedlings (Heydari et al., 1997). All isolates have been efficient cotton root colonizers and have been capable of suppressing pathogen-induced stress. All five isolates were used in microcosm experiments and one isolate (D1) was tested in the field.

In microcosm experiments, population sizes of most of the bacterial isolates in the rhizosphere of cotton seedlings in soils treated with each of the three herbicides were significantly ( $P < 0.05$ ) lower than those in the untreated soils 2 weeks after sowing.

The ability of all three test herbicides to reduce isolate D1 population in the rhizosphere declined with time over a 4 week period of monitoring. The population of the bacterium recovered from roots in the herbicide-treated soils was significantly ( $P < 0.05$ ) lower than those recovered from controls after 1 and 2 weeks but were equivalent to the controls 3 and 4 weeks after sowing (Heydari et al., 1997).

Results of the field experiments were similar to those of the microcosm experiments (Tables 30.3 and 30.4). Pendimethalin and prometryn caused significant ( $P < 0.05$ ) decrease in the D1 population in the rhizosphere 15 and 25 days after sowing at Safford (Table 30.3). Trifluralin had no significant effect on the D1 population at this location (Table 30.3). Pendimethalin and prometryn

**TABLE 30.3**  
**Population Sizes ( $\times 10^6$  cfu  $g^{-1}$  Root) of *Burkholderia cepacia* (Isolate D1) in the Rhizosphere of Cotton Seedlings Grown in Soils Treated or Not Treated with Pendimethalin, Prometryn or Trifluralin, 15, 25, and 50 Days after Sowing in Safford Field Experiment**

| Treatment              | Time (Days after Sowing) |              |             |
|------------------------|--------------------------|--------------|-------------|
|                        | 15                       | 25           | 50          |
| Control (no herbicide) | 5.3 (2.5) a              | 3.0 (2.2) a  | 2.2 (1.8) a |
| Pendimethalin          | 2.6 (1.8) b              | 1.4 (1.3) b  | 1.7 (1.1) a |
| Prometryn              | 2.4 (1.1) b              | 1.8 (1.3) b  | 1.8 (1.4) a |
| Trifluralin            | 4.1 (2.2) a              | 2.4 (1.9) ab | 1.9 (1.3) a |

*Notes:* Each mean is an average of four values obtained in one experiment with four replicates. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

**TABLE 30.4**  
**Population Sizes ( $\times 10^6$  cfu g<sup>-1</sup> Root) of *Burkholderia cepacia* (Isolate D1) in the Rhizosphere of Cotton Seedlings Grown in Soils Treated or Not Treated with Pendimethalin, Prometryn or Trifluralin, 15, 25, and 50 Days after Sowing in Tucson Field Experiment**

| Treatment              | Time (Days after Sowing) |             |             |
|------------------------|--------------------------|-------------|-------------|
|                        | 15                       | 25          | 50          |
| Control (no herbicide) | 7.2 (4.2) a              | 2.8 (2.0) a | 1.0 (0.8) a |
| Pendimethalin          | 3.0 (0.9) b              | 1.3 (0.8) b | 0.8 (0.5) a |
| Prometryn              | 2.0 (1.5) b              | 1.1 (0.9) b | 0.7 (0.5) a |
| Trifluralin            | 5.6 (2.9) a              | 1.6 (1.0) b | 1.0 (0.6) a |

*Notes:* Each figure (mean value) is an average of four values obtained in one experiment with four replicates. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

caused significant ( $P < 0.05$ ) decrease in the D1 population in the rhizosphere 15 and 25, but not 50 days after sowing at Tucson (Table 30.4). The trifluralin-induced decline in the D1 population was significant only 25 days after sowing (Table 30.4). Isolate D1, which was originally recovered from cotton plants in the field, may have developed tolerance to trifluralin as a result of the continuous exposure to this herbicide in the field.

### 30.7 EFFECT OF HERBICIDES ON BIOCONTROL ACTIVITY OF BACTERIA IN THE RHIZOSPHERE

Our earlier finding that soil atmospheric composition can modulate biocontrol activity of selected bacteria (Kim and Misaghi, 1996) spurred us to examine the impact of other soil factors on the activity of biocontrol bacteria in the rhizosphere. We, therefore, examined the impact of three widely used herbicides, pendimethalin, prometryn, and trifluralin on the efficacy of isolate D1 (a biocontrol bacterium) to reduce the severity of *R. solani*-induced seedling death. Isolate D1 has been capable of reducing the incidence of *R. solani*-induced seedling death in the field (Zaki et al., 1998). In both field and microcosm experiment the efficacy of isolate D1 was reduced in the presence of two of the three test herbicides. In Safford field experiment isolate D1 reduced seedling death severity significantly ( $P < 0.05$ ), compared to the control (not treated with D1), 15, 25, and 50 days after sowing only in nonherbicide treated plots and in plots treated with trifluralin, and not in plots treated with pendimethalin and prometryn (Table 30.5). In Tucson field experiment, biocontrol bacterium (isolate D1) reduced cotton seedling death in plots not treated with herbicides and in those treated with trifluralin significantly, compared to the control (not treated with D1) 15, 25, and 50 days after sowing (Table 30.6). Pendimethalin and prometryn both decreased the efficacy of isolate D1 in reducing cotton seedling death significantly ( $P < 0.05$ ), 25 and 50 days after sowing in Tucson experiment (Table 30.6). In contrast to our findings, the herbicides pendimethalin and metribuzin have been reported to enhance biocontrol activity of *Streptomyces corchorusii* and *S. mutabilis* in greenhouse tests (Elshanshoury et al., 1996).



**TABLE 30.5**  
**Stand Count (The Number of Emerged Seedlings)**  
**in Soils Treated with Each Test Herbicide, Inoculated**  
**with *Rhizoctonia solani* and/or Biocontrol Bacterium**  
**(*Burkholderia cepacia*, Isolate D1) 15, 25, and 50 Days**  
**after Sowing in Safford Field Experiment**

| Treatment                             | Time (Days after Sowing) |           |           |
|---------------------------------------|--------------------------|-----------|-----------|
|                                       | 15                       | 25        | 50        |
| <i>R. solani</i> alone                | 166(15) b                | 126(12) b | 105(12) b |
| <i>R. solani</i> + D1                 | 217(23) a                | 193(19) a | 184(15) a |
| <i>R. solani</i> + D1 + pendimethalin | 153(53) b                | 121(50) b | 98(40) b  |
| <i>R. solani</i> + D1 + prometryn     | 163(13) b                | 127(15) b | 85(26) b  |
| <i>R. solani</i> + D1 + trifluralin   | 202(23) a                | 186(20) a | 161(23) a |

*Notes:* Each figure (mean value) represents the average number of emerged seedlings in one plot (one replicate) sowed with 400 seeds. Each figure is average of four values obtained in one experiment with four replicates. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

**TABLE 30.6**  
**Stand Count (The Number of Emerged Seedlings)**  
**in Soils Treated with Each Test Herbicide, Inoculated**  
**with *Rhizoctonia solani* and/or Biocontrol Bacterium**  
**(*Burkholderia cepacia*, Isolate D1) 15, 25, and 50 Days**  
**after Sowing in Tucson Field Experiment**

| Treatment                             | Time (Days after Sowing) |            |           |
|---------------------------------------|--------------------------|------------|-----------|
|                                       | 15                       | 25         | 50        |
| <i>R. solani</i> alone                | 101(13) b                | 87(11) b   | 79(18) b  |
| <i>R. solani</i> + D1                 | 171(29) a                | 164(29) a  | 158(25) a |
| <i>R. solani</i> + D1 + pendimethalin | 117(33) b                | 107(32) b  | 102(32) b |
| <i>R. solani</i> + D1 + prometryn     | 168(51) a                | 121(25) ab | 110(31) b |
| <i>R. solani</i> + D1 + trifluralin   | 180(17) a                | 157(19) a  | 151(21) a |

*Notes:* Each figure (mean value) represents the average number of emerged seedlings in one plot (one replicate) sowed with 400 seeds. Each figure is average of four values obtained in one experiment with four replicates. Means values followed by the same letter in each column are not significantly different at the 0.05 probability level ( $P > 0.05$ ) according to the Duncan multiple range test. Values in parentheses are standard deviations.

### 30.8 MECHANISM OF HERBICIDE-MEDIATED ALTERATIONS IN PATHOGEN-INDUCED STRESS

The mechanism of the observed herbicide-mediated change in the intensity of pathogen-induced stress is not known. The phenomenon may be due to the effect of herbicides on the plant, on the pathogen, on the activity of indigenous microbial competitors and/or on the interactions among these entities.

Herbicides may cause changes in plant root physiology such as root exudation (Brown and McCarter, 1976; Elshanshoury et al., 1996). These changes, in turn, may alter microbial community structures in the rhizosphere in ways which may encourage or discourage the development of competitors of an introduced biocontrol bacterium. Such changes may enhance or depress the activity of biocontrol bacteria. Herbicides may alter the intensity of pathogen-induced stress by changing plant resistance levels to pathogens. Starratt and Lazarovits (1996) showed that the application of dinitroaniline herbicides induced resistance in tomato seedlings to pathogen, *Fusarium oxysporum* f. sp., *lycopersici*. Herbicides also may cause changes in crop plants, which may influence the outcome of plant–pathogen interactions (Heydari et al., 1997). Herbicides have been reported to cause alteration in growth, lignin-containing substances, B-glucoside, wax layer on leaves, and in the release of glucose from roots (Starratt and Lazarovits, 1996; Wyse et al., 1976). While the height, biomass, and root densities of cotton seedlings grown in soils treated with pendimethalin or prometryn were generally lower than those of control (untreated soil) in our studies, the differences were not statistically significant ( $P > 0.05$ ), indicating that physical characteristics of cotton seedlings were not affected by test herbicides.

The observed herbicide-mediated changes in the intensity of pathogen-induced stress may be due to the effect of herbicides on the pathogen (Altman and Campbell, 1977; Black et al., 1996). Such effect may be stimulatory or inhibitory. For example, in the *R. solani*–sugar beet combination, herbicide cycolate may have interfered with the growth of the fungus and at the same time may enhance root exudates (Altman and Campbell, 1977). In such cases, the impact of the herbicide on the intensity of pathogen-induced stress is determined by the balance of stimulatory and inhibitory effects. In our study, the growth of *R. solani* in in vitro condition was not significantly affected by pendimethalin, prometryn, or trifluralin.

Herbicide-mediated alterations in pathogen-induced stress may also be due to the effect of herbicides on indigenous microbial antagonists of pathogens (Elshanshoury et al., 1996; Heydari et al., 1997). The observed absence of soil-borne diseases in some fields in the presence of susceptible hosts and virulent pathogens is most likely due to the presence of indigenous microbial antagonists of the pathogen (Heydari et al., 1997). As pointed out earlier, we found that herbicides, pendimethalin, prometryn, and trifluralin decreased the populations of some biocontrol bacteria in the rhizosphere of cotton.

Finally, herbicides may change the intensity of pathogen-induced stress by interfering with the activity of fungicides used to curb the pathogenic activity of pathogens (Awadalla and El-Refaie, 1994; Hans and Dodan, 1982; Moustafa-Mahmoud et al., 1993; Youssef et al., 1987). Application of fluchlovalin and alachlor to the soil altered the effectiveness of fungicides to control the cowpea damping-off (Hans and Dodan, 1982). Application of nurflurazon, pendimethalin, fluometuron, prometryn, fomesafen, and oxyfluorfen to the field soil significantly reduced the efficacy of fungicides, tolclofos-methyl, penycuron, carboxin, flutonalit, metalaxyl, and chloroneb against cotton seedling diseases (Youssef et al., 1987). In contrast the antifungal activity of fungicides, captan, and mounsrin was shown to be increased in the presence of herbicides, paraquat, and simazine (Awadalla and El-Refaie, 1994).

While plants, pathogens, and antagonistic microorganisms are perhaps the primary target of herbicides, the possibility of other herbicide-mediated changes cannot be overlooked. One such possibility is an alteration of the microclimate as a consequence of removal of weeds as suggested by Awadalla and El-Refaie (1994). We agree with Altman and Campbell (1977) that no single factor may be solely responsible for the observed herbicide effect on the outcome of plant–pathogen interactions.

### 30.9 MECHANISM OF HERBICIDE-MEDIATED CHANGES IN BIOCONTROL ACTIVITY OF BACTERIA

The observed herbicides interference with the biocontrol activity of isolate D1 (Heydari et al., 1997) and *Streptomyces* sp. (Elshanshoury et al., 1996) is most likely due to the effect of herbicides on the biocontrol agents. The sensitivity of microorganisms to herbicides has been demonstrated (Atlas et al., 1978; Forlani et al., 1995; Heydari et al., 1997). Results of our preliminary studies also have shown that the growth of isolate D1 in a liquid medium was reduced by 48%, 44%, and 32%, 24 h after exposure to pendimethalin, prometryn, or trifluralin. Herbicide-mediated change in the performance of biocontrol agents may also be a consequence of herbicide-induced change in pathogen and plant, which was discussed earlier. For example, increased activity of a pathogen (in terms of growth and aggressiveness) in the presence of a herbicide may tip the balance in favor of the pathogen, reducing the effectiveness of the biocontrol agent. Herbicides may also provide favorable environment for some indigenous competitors of the biocontrol agent. Herbicide-induced changes in plant may cause alterations in microbial community structures, encouraging or discouraging biocontrol activity. Another possibility is the herbicide-mediated shift in the quality and the quantity of root exudates and/or border cells in plants (Wilcox, 1996), which can alter microbial community structure. Finally, herbicides may affect cross-communication among microorganisms and plant roots, causing drastic changes in the activity of the introduced biocontrol agents and their indigenous competitors. Cross-communication among microorganisms and between plants and microorganisms has been demonstrated (McKenny et al., 1995; Pierson and Pierson, 1996).

### 30.10 CONCLUSIONS

Results of studies presented here clearly show that herbicides, which are being used extensively throughout the world, (a) may alter severity of pathogen-induced stress, (b) may affect the efficacy of biocontrol agents used to curb pathogen-induced stress, and (c) microorganisms are differentially sensitive to herbicides. In our study trifluralin did not cause any significant change in the incidence of cotton seedling death both in the microcosm and in the field experiments. Rhizosphere-associated microorganisms including *R. solani*, may have developed some levels of tolerance to this herbicide due to its wide-spread and long-term use in cotton fields.

The results presented here have important implications for disease management. This is particularly true for seedling diseases in which plants are vulnerable to attack by pathogens when most herbicides may still be present in the soil at biologically active levels. Selection of herbicide must be done cautiously in areas where plant diseases are important. Ideally, available herbicides for a particular crop need to be screened for their effect on pathogen-induced stress and on the biocontrol activity of selected biocontrol agents. Since bacterial isolates are differentially sensitive to herbicide, it may also be possible to first select a herbicide and then to choose a biocontrol agent whose biocontrol activity is not adversely affected by the presence of the selected herbicide. It may also be possible to genetically construct bacterial isolates with increased tolerance to herbicides.

The sensitivity of some microorganisms, including biocontrol-active *B. cepacia* to herbicides as was reported by several workers cited in this chapter and herbicides ability to increase the incidence of some diseases as was reported by several workers cited in this chapter. Provide additional support in favor of the concept of integrated pest management (IPM) strategy. This strategy encourages crop specialists to base their decision regarding the use of a pesticide not only on the effectiveness of the pesticide against the target pest but also on its potential impact on all crop pests in the region. The selection of an ideal pesticide (one which is not harmful to the crop, to the nontarget microorganisms, and to the beneficial insects) is difficult because of the number and diversity of pests involved in any one crop in any region. For example, in addition to insects and weeds, cotton seedlings are damaged by a number of soilborne pathogens besides *R. solani*. Despite these problems,

IPM strategies need to be developed for specific crops in specific regions. The development of IPM requires knowledge of the impact of a selected pesticide not only on its intended target but also on plants as well as on beneficial and harmful microorganisms and insects. Application of IPM strategy also requires major changes in agricultural development policies and institutions as was reported by some workers cited in this chapter.

While many herbicides are readily biodegraded within a week, others may remain active for up to 2 months following application to the soil. We found that the ability of all three test herbicides to reduce biocontrol bacterium (isolate D1) populations in the rhizosphere decreased with time, perhaps due to degradation of the herbicides. However, all three herbicides tested in Safford field experiment were biologically active up to 25 days after sowing. The persistence of herbicides in the soil depends on several factors including soil moisture, temperature, pH, organic matter content, clay content, and chemical structure of the herbicides as was reported by some workers cited in this chapter.

With the increasing world population, providing food for fast-growing populations is becoming a critical issue. A sustainable agricultural system as the most important food source is therefore extremely important and should be taken very seriously. Harmful pests (insects, pathogens, weeds) are among the major stress-inducing agents for the plants and the most important limiting factors for the agricultural yield and production. Nowadays, biological control is considered as one of the most important strategies for management of plant pests, including harmful pathogens. To achieve a successful biological control in the field, studying the interactions among environmental factors and biocontrol active microorganisms is important and critical. Such studies can potentially help scientists and farmers to promote and increase the efficiency of biological control agents in the field, increase the yield and productivity of crops and plants, and protect the environment and biological resources.

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# 31 Stress in Plants and Crops Induced by Fungal Pathogens

*Asghar Heydari and Giorgio M. Balestra*

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## 31.1 INTRODUCTION

Pathogenic fungi can attack a very wide range of plants, and by doing so can cause very serious losses to agricultural and horticultural crops (Agrios, 1988; Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari, 2007; Heydari et al., 2005; Heydari and Misaghi, 2003; Mayer et al., 2001; Ortoneda et al., 2004; Park et al., 2005; Scully and Bidochka, 2006; St. Leger et al., 2000; Zaki et al., 1998). A great deal of research has been carried out to study the pathogens, but very little consideration has been given to the mechanisms involved in the pathogenic process of these types of organisms. As a result of fungal pathogen attack, different stressful conditions are induced and created in plants and crops. In this review, an attempt will be made to describe and discuss these stresses.

In the case of fungal pathogen attack, severe damage is caused to the host, but this damage does not necessarily lead to death of the host plant (Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari, 2007; Heydari et al., 2005; Heydari and Misaghi, 2003). The attack by necrotrophic fungi, in which host cells are killed, presents a slightly different situation (Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari, 2007). Following location of the host, fungi in some way attach to the plant (Alexopolous and Mimms, 1996). The pathogen, after attachment, must penetrate the outer layers of the host, by mechanical breaching of the defense barriers or by using chemical mechanisms to disrupt physical barriers, for example, by the use of lytic enzymes or through existing openings such as stomata (Alexopolous and Mimms, 1996; Doehlmann et al., 2006; Heydari, 2007; Heydari et al., 2005).

Following penetration of the host's outer layers, the pathogen either enters into or fuses with the host tissue, permitting the withdrawal of nutrients from the host plant (Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006). In optimal parasitism, the attacking organism neutralizes the host defense responses so that a continued coexistence is possible, without death of the host cells. This process of neutralization involves a series of complex reactions, in which levels of host hormones (growth substances) are modified or hormones are secreted by the attacking organisms (Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari, 2007). The pathogen creates a local environment, in which the parasite becomes a sink for the nutrients present in the host, without, however, completely depleting host resources (Alexopolous and Mimms, 1996). Fungal pathogens frequently produce toxins that have an adverse effect on the host, without necessarily killing it (Brodhagen and Keller, 2006; Daly and Knoche, 1982; Dubrin, 1981; Mitchel, 1984). In extreme cases, parasite toxins kill host cells or tissues.

An important part of the pathogen–host interaction is the genetic mechanisms of compatibility or incompatibility (Belkhadir et al., 2004; Doehleemann et al., 2006; Dunkle, 1984; Feldbrugge et al., 2004). This involves the ability of the pathogen to recognize the host and the host to sense the presence of the pathogen. Incompatibility, in which the host recognizes and rejects the pathogen, is part of the resistance mechanism. The ability of the pathogen to recognize its host is also a very significant part of the attack process (Belkhadir et al., 2004; Doehleemann et al., 2006; Dunkle, 1984; Feldbrugge et al., 2004).

The number of fungi is extremely large and the number of pathogens among them comprises probably thousands of species (Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari, 2007; Heydari et al., 2005; Heydari and Misaghi, 2003; Mayer et al., 2001; Ortoneda et al., 2004; Park et al., 2005). It is characteristic of most pathogenic fungi to show a great deal of species specificity. Most of the biotrophic fungi are able to infect only a very limited number of plant species, whereas the necrotrophic fungi are far more versatile and are often able to infect hundreds of plant species (Alexopolous and Mimms, 1996; Chen and Dickman, 2005; Doehlmann et al., 2006; Heydari and Misaghi, 2003; Mayer et al., 2001; Ortoneda et al., 2004; Park et al., 2005).

Fungal plant pathogens and the infection and diseases they cause in host plants can result in various stressful conditions. They may affect the physiological functions of plants and cause serious damage and disruptions to these functions. Almost all essential physiological functions of plants, including photosynthesis, translocation of water and nutrients, transpiration, respiration, permeability of cell membrane, and transcription and translation, can be affected by fungal pathogens (Allakhverdieva et al., 2001; Antunes and Sfakiotakis, 2000; Camejo et al., 2005; Crafts-Brander and Salvucci, 2002; Hancock and Huisman, 1981; Manners and Scott, 1983). As a result, serious stressful conditions may be induced and created in plants. In this chapter, the pathogenesis and the ways that fungal pathogens attack and cause diseases in their hosts will be first discussed and then their stressful impacts on the structures and functions of plants will be reviewed.

## 31.2 ATTACHMENT OF FUNGAL PATHOGENS TO HOST PLANTS

The manner in which fungal pathogens reach their host plants depends on the part of the host that is attacked (Alexopolous and Mimms, 1996). If above-ground parts of the plants are attacked by pathogens, frequently the fungal pathogen reaches them in a fortuitous manner, due to the accidental spread of the fungal structural elements such as mycelium or spores, by wind, rain, or other environmental factors (Agrios, 1988; Podila et al., 1993). In some cases, the infecting fungal pathogen may be carried by insects or other organisms, but again, contact occurs in an accidental fashion. When plant roots are attacked, there is often an actual active mechanism by which the fungus reaches the host (Agrios, 1988; Alexopolous and Mimms, 1996). In some cases, fungal mobile structures such as zoospores are attracted to the roots by chemotactic mechanisms. In other instances, the infecting pathogen may reach the root accidentally or it may be carried there by organisms present in the soil (Agrios, 1988).



Usually, fungal infection of a host plant is preceded by germination of a fungal spore (Alexopolous and Mimms, 1996). The spore lands on the surface of its host, germinates, and then one of a number of alternative pathways is followed. In the case of zoospores, after encystment and germination, growth is towards the host, probably by a chemotactic mechanism, although much remains to be elucidated in this respect (Alexopolous and Mimms, 1996). Hyphae may enter the host through natural openings, such as stomata, lenticels, or hydathodes, or through existing lesions or wounds in the surface. Sometimes the hyphae form an appressorium, a rigid structure, often containing melanin, which sticks to the surface of the host (Agrios, 1988; Alexopolous and Mimms, 1996; Dunkle, 1984; Francis et al., 1996; Hoch and Staples, 1987; Podila et al., 1993; Thines et al., 2000; Veneault-Fourrey et al., 2006).

The appressorium sticks to the surface of the host with the aid of adhesive compounds, which include glycoproteins, polysaccharides, polymers of hexosamines and xylans, and perhaps also lipids (Agrios, 1988). The nature of the adhesive can be quite variable, depending on the host and the infecting fungus (Thines et al., 2000; Veneault-Fourrey et al., 2006). Eventually, the appressorium develops sufficient turgor pressure so that a mechanical breach of the cuticle is possible (Hoch and Staples, 1987; Thines et al., 2000; Veneault-Fourrey et al., 2006). The cuticle on the surface of the host may be softened by the action of enzymes, such as cutinase, enabling penetration.

### 31.3 HOST PLANT PENETRATION BY FUNGAL PATHOGENS USING MECHANICAL FORCES

Fungal Plant pathogens are very small and microscopic organisms that cannot generally apply a voluntary force to plant surface (Alexopolous and Mimms, 1996). In order to enter plant tissues and cells, they need to be equipped with some mechanical structures by which they reach and enter their host plants (Goodman et al., 1986; Isaac, 1992; Misaghi, 1982; Strange, 2003). The penetration of fungal pathogens to the tissue of their host has been well studied (Alexopolous and Mimms, 1996; Goodman et al., 1986; Isaac, 1992; Strange, 2003). Pathogenic fungi are facilitated with some mechanical structures such as haustorium and appressorium, which can be used when these pathogens penetrate host plants (Agrios, 1988; Alexopolous and Mimms, 1996; Thines et al., 2000; Veneault-Fourrey et al., 2006).

The fungal haustorium is a well-recognized structure, which is basically an extension of part of a fungal hypha that penetrates into the cell or cells of the host, without lesion or lysis of the host cell wall (Alexopolous and Mimms, 1996). It can be likened to the finger of a glove, which deforms the cell wall of the host cell, but does not damage it. The fungal haustorium is well described and it is sufficient here to cite the general literature (Agrios, 1988; Alexopolous and Mimms, 1996; Thines et al., 2000; Veneault-Fourrey et al., 2006).

The fungal haustorium is able to obtain nutrients from the host cells (Alexopolous and Mimms, 1996). Mechanical forces and stress induced by them in plants attacked by fungal pathogens are also mediated by appressorium, which is a swollen tip of a hyphae or germ tube that facilitates attachment and penetration of the host by the fungus (Agrios, 1988; Alexopolous and Mimms, 1996). Appressoria are either melanized, such as those of *Colletotrichum* and *Magnaporthe*, or non-melanized such as that belongs to *Erysiphe*, which is an obligate parasite and the causal agent of powdery mildew disease on many plants (Agrios, 1988; Alexopolous and Mimms, 1996).

After penetration of the host plants, a considerable amount of mechanical force is exerted on host tissues by fungal pathogens. Through increased pressure created by this force, plant tissues, cell walls, and cuticle are expanded and pushed out, and finally break down (Alexopolous and Mimms, 1996; Goodman et al., 1986; Isaac, 1992; Strange, 2003). Once a pathogenic fungus has entered a plant cell, it usually secretes increased amounts of enzymes that complete the process of penetration and assist the pathogen to establish and settle down in the host plant (Alexopolous and Mimms, 1996; Goodman et al., 1986; Isaac, 1992; Misaghi, 1982; Strange, 2003).

### 31.4 HOST PLANT PENETRATION BY FUNGAL PATHOGENS BY THE AID OF ENZYMES

In addition to mechanical penetration, fungal pathogens also use biochemical weapons including different enzymes to enter plant tissues (Francis et al., 1996; Idnurm and Howlett, 2002; Jennings et al., 1998; Kolattukudy, 1981, 1985; Koller et al., 1982, 1995; Kosuge and Nester, 1984; Mohavedi and Heale, 1990; Thines et al., 2000; Tian et al., 2004; Van Kan, 2006; Ten Have et al., 2004). Initial penetration may be through natural openings, such as stomata, lenticels, or cracks in the surface of the host. However, even when this occurs, enzymes are subsequently used to continue penetration. Such penetration is achieved by the formation of several groups of enzymes (Francis et al., 1996; Jennings et al., 1998; Kolattukudy, 1981, 1985; Koller et al., 1995). The first group comprises those that degrade the surface layers of the host, such as layers of waxes, cutins, and suberins (Francis et al., 1996; Koller et al., 1982, 1995).

Degradation of cell wall substrates is the most important mode of action and mechanism of enzymes that enable fungal pathogens to penetrate plant tissues (Agrios, 1988; Idnurm and Howlett, 2002). The most thoroughly researched of these enzymes are the cutinases. Although the evidence of the function of cutinases in fungal penetration is not unambiguous, there is a great deal of evidence to show that they do play a role in penetration into the host (Francis et al., 1996; Koller et al., 1995).

The next group of enzymes secreted by fungi that are well researched and extensively described are those involved in the degradation of pectins (Agrios, 1988; Kolattukudy, 1981, 1985). A wide range of enzymes varying in activity, specificity, distribution, and function are known. One way of discriminating among them is by a definition of their activity. On the one hand, there are the polygalacturonases, which cleave the galacturonide bonds in the pectin skeleton (Alexopolous and Mimms, 1996). These enzymes may be endo- or exo-polygalacturonases or in some cases endo- or exo-methyl polygalacturonases (Alexopolous and Mimms, 1996; Mohavedi and Heale, 1990). Although having an entirely different mode of action, the pectin lyases or pectin trans-eliminases also break down the galacturonide bond of the pectin (Agrios, 1988). The number of these enzymes is very large, and they usually exist as a number of isozymes that are coded by a series of genes, the expression of which depends on the pathogen, its stage of development, and the host (Agrios, 1988).

However, their importance in pathogen penetration into the host is not in doubt, although often, the specific isozyme that is important has not been determined. Frequently, the action of the polygalacturonases is preceded by the action of the pectin methyl esterases, which cleave the methyl group attached to the carboxylic acid present in pectins (Mohavedi and Heale, 1990). Again, endo- and exo-pectin methyl esterases are known that are coded by a large number of genes. Many isozymes are known and in general it can be stated that the pectin methylesterases are an essential part of penetration of the fungal pathogen into its host. Also in this case, the literature is extensive and will not be reviewed here.

Other enzymes present in many pathogens are the cellulases and xylanases, which act directly on the glycosidic bonds of cellulose or on those of arabinoxylans present in the plant cell wall (Agrios, 1988; Alexopolous and Mimms, 1996). These enzymes have been less well described and their role in fungal penetration is less clear. Although the cell wall of many plant cells is lignified, the degradation of lignin is probably not a crucial step in fungal penetration (Agrios, 1988). Lastly, proteases produced by fungal pathogens must be mentioned. These are apparently involved in pathogenicity and may be responsible for the initial damage to host cells (Alexopolous and Mimms, 1996; Doehleemann et al., 2006).

Overall, the concept emerging from the studies of these enzymes reveal that fungi use an array of enzymes to break down host cell walls to separate host tissue and to permit penetration into host tissue, and, in addition, are often able to inactivate or kill the host cells, thereby liberating nutrients that are then available for growth and development of the fungal pathogen. In addition to fungal enzymes, hormones formed by the pathogen also often play a role in redirecting the flow of nutrients toward the site of infection, thereby improving the sink from which the pathogen obtains its nutrients (Agrios, 1988).

### 31.5 PATHOGENESIS OF FUNGAL PATHOGENS ON HOST PLANTS BY TOXIN PRODUCTION

In phytopathology, the term “toxin” is reserved for toxic chemicals (chemopathogens) that are able to cause effects similar to those of disease symptoms induced by microorganisms (Gardiner et al., 2005; Scheffer, 1983). This may be confused with two other names: phytotoxins (for any product of a living organism toxic to plants) and mycotoxins (produced by several fungi in infected seeds, feeds, or foods and capable of causing illnesses of varying severity and death to animals and humans that consume such substances).

Most microbe toxins are low molecular weight compounds with diverse structures that act as positive agents of virulence or pathogenicity (Brodhagen and Keller, 2006; Daly and Deverall, 1983; Gardiner et al., 2005; Nishimura and Komoto, 1983; Palmer et al., 2004; Scheffer, 1983). Pathogenic fungi can produce toxins in infected plants as well as in culture medium. The toxin is produced only in the plant or under specific inductive conditions are much less likely to have been discovered. Toxins are usually effective in very low concentrations, target the cellular membranes by affecting the permeability, inactivate or inhibit enzymes, and inhibit or disturb the signal transduction (Brodhagen and Keller, 2006; Dally and Deverall, 1983; Mitchel, 1984; Nishimura and Komoto, 1983; Scheffer, 1983).

#### 31.5.1 NON-HOST-SPECIFIC TOXINS

Toxins produced by phytopathogenic microbes have been shown to produce all or part of the disease syndrome, not only in the host plant but also in other species of plants that are not normally attacked by the pathogen in nature (Scheffer, 1983). These toxins increase the extent of disease caused by a pathogen but are not essential for the pathogen to cause disease (Scheffer, 1983). By definition, non-host-specific toxins are not primary determinants of host range. One of the typical fungal non-host-specific toxins is fusicoccin, which is a diterpenoid glycoside synthesized via the mevalonic acid pathway and is produced by *Fusicoccum amygdali*, causing the wilting syndrome in peach and almond trees (Agrios, 1988; Brodhagen and Keller, 2006; Scheffer, 1983). The fusicoccin toxin causes the opening of the stomata and increased transpiration and subsequent wilting in plants. The site and mode of action of this toxin is the direct activation of plant  $H^+$ -ATPase and reversible interaction between its C-terminal region and regulatory 14-3-3. The 14-3-3 proteins are present in all eukaryotes and act as regulators in various signal transduction pathways (Brodhagen and Keller, 2006).

Different *Fusarium* species produce a complex of several toxins including fusaric acid (5-butylpicolinic acid), fumonisin B<sub>1</sub> (produced by *F. moniliform* and by *Alternaria alternata* f. sp. *lycopersici* as AAL-toxin), trichothecene vomitoxin-deoxynivalenol (DON), zearalenone (ZEN), T-2 toxin, etc. (produced by *F. graminearum* and other *Fusarium* species). These toxins are phytotoxic to a broader range of plants (Agrios, 1988). A number of other NHS-toxins such as oxalic acid have been isolated from phytopathogenic fungi (*sclerotium* and *sclerotinia*) and many others.

#### 31.5.2 HOST-SPECIFIC (SELECTIVE) TOXINS

A host-specific toxin is a substance produced by a pathogenic microbe that, at physiological concentrations, is toxic to the hosts of that pathogen and shows little or no toxicity against non-susceptible plants (Brodhagen and Keller, 2006; Mitchel, 1984; Nishimura and Komoto, 1983; Scheffer, 1983). In general, host-specific (selective) toxins (HSTs) are determinants of host range or specificity in that plant species, varieties, or genotypes. HSTs have been critical factors in two major epidemics of crops in the United States in the twentieth century, including the southern corn leaf blight epidemics of 1970 that destroyed about 15% of that year's crop (Mitchel, 1984). They are also important factors in several other economically significant diseases. In general, all known HSTs are

produced by certain fungi. Even among the fungi, most known HSTs are produced by species or pathotypes in just two genera: *Cochliobolus* (also known by the old and new names for its imperfect stage, *Helminthosporium* or *Bipolaris*, respectively) and *Alternaria* (Brodhagen and Keller, 2006; Mitchel, 1984; Nishimura and Komoto, 1983; Scheffer, 1983).

Victorin is one of the first host-specific toxins discovered, and its remarkable toxic effects have been recorded (Agrios, 1988). Its structure is a polypeptide linked to a nitrogen-containing sesquiterpene and is produced by *Cochliobolus victoriae*, which causes foot and root rot and leaf blight in certain oat varieties (Agrios, 1988; Brodhagen and Keller, 2006). Plant phenotypical reactions and stresses caused by this toxin include some general changes in the physiology of host that are common to infectious plant diseases. The site and mode of action of this toxin is the major 100-kD victorin-binding protein that has been purified, and its gene encodes the pyridoxal-phosphate-containing P subunit of glycine decarboxylase (GD) (Brodhagen and Keller, 2006).

Another important toxin in this group is T-toxin (HMT-toxin). It is produced by race T isolate of *C. heterostrophus*, the causal agent of the southern corn leaf blight that is believed to be one of the most serious diseases in the recent history of plant pathology (Agrios, 1988; Brodhagen and Keller, 2006). Race O of *C. heterostrophus*, however, which does not produce T-toxin, is a minor pathogen of corn regardless of cytoplasm. Site and mode of action of T-toxin is active at about 10 nM against Tcms maize and at about 10  $\mu$ M against maize with normal cytoplasm. Mitochondria in vitro and in situ are quickly, specifically, morphologically, and biochemically affected by T-toxin (Agrios, 1988; Brodhagen and Keller, 2006).

HC-toxin is another host-specific toxin, which is a cyclic tetrapeptide produced by *C. carbonum* race 1, a causative agent of the Northern corn leaf spot (Agrios, 1988; Brodhagen and Keller, 2006). This toxin inhibits root growth of compatible maize, but is not toxic to cells. Current evidence indicates that the site of action of HC-toxin is histone deacetylase (HD), an enzyme that reversibly deacetylates the core histone (H3 and H4) while they are assembled in chromatin (Brodhagen and Keller, 2006). Acetylation and deacetylation of the core histones alter the inducibility and suppressibility of certain classes of genes. Current research is aimed at understanding how the inhibition of HD activity promotes the infection of maize by *C. carbonum* race 1.

Finally, AAL-toxin is the last host-specific toxin discussed in this section. The structure of this toxin is related to sphinganine and is produced by *Alternaria alternata* f. sp. *lycopersici* as AAL-toxin and by *Fusarium moniliform* as fumonisin B<sub>1</sub> toxin (Agrios, 1988; Brodhagen and Keller, 2006). The AAL-toxin is essential for the pathogenesis of AAL in tomato, because the toxin-deficient mutants cannot infest healthy compatible tomato leaves. However, all strains of *F. moniforme* are not pathogenic on the AAL compatible tomato isolate, indicating that the toxin produced by *F. moniforme* is not sufficient for virulence on tomato (Brodhagen and Keller, 2006). Plant phenotypical reactions, toxicity, and resistance of this toxin indicates that it acts as a phytotoxin against plants. The AAL-toxin is toxic to all tissues of sensitive tomato cultivars at low concentrations and induces apoptosis in sensitive tomato lines (Brodhagen and Keller, 2006).

### 31.6 PATHOGENESIS OF FUNGAL PATHOGENS ON HOST PLANTS BY GROWTH REGULATORS

Growth regulators are naturally occurring compounds in plants and act in very low concentrations (Agrios, 1988; Ashraf and Foolad, 2007; Cessna et al., 2000; Hamer and Holden, 1997; Mur et al., 2006; Tsitsigiannis and Keller, 2006; Viaud et al., 2002; Walters et al., 2006; Wang et al., 2003). The production and occurrence of growth regulators are usually changed and altered after fungal pathogens attack and infect plants. The most important growth regulators are discussed below.

Auxin, which is also called IAA, is one of the most important growth regulators involved in fungal pathogenicity (Agrios, 1988; Ashraf and Foolad, 2007; Cessna et al., 2000; Hamer and Holden, 1997). Studies have shown that IAA levels are changed in many diseased plant tissues. Several fungal plant diseases such as corn smut (*Ustilago maydis*) and clubroot of cabbage (*Plasmodiophora*

*brassicae*) show a typical gall symptom due to the increased levels of IAA (Agrios, 1988; Mur et al., 2006; Tsitsigiannis and Keller, 2006; Viaud et al., 2002). Many other fungal pathogens are also capable of production of IAA.

Gibberellin is another growth regulator that is directly related to fungal pathogen infection (Agrios, 1988; Cessna et al., 2000; Hamer and Holden, 1997; Mur et al., 2006). A century ago, rice farmers in Asia noticed some exceptionally tall seedlings growing in their paddies (Agrios, 1988). Before these rice seedlings could mature and flower, they grew so tall and spindly that they toppled over. In Japan, this aberration in growth pattern became known as *bakanae* (foolish seedling disease) of rice. In 1926, Kurosawa, a Japanese scientist discovered that the disease was caused by a fungal pathogen, *Gibberella fujikuroi* (Agrios, 1988). By the 1930s, Japanese scientists had determined that this fungus produced hyper-elongation of rice stems by secreting a chemical, which was given the name gibberellin. Gibberellins are normal constituents of green plants and also produced by several other microorganisms. The best known gibberellin is gibberellic acid. In the past years, scientists have identified more than 80 different gibberellins, many of them occurring naturally in plants. Spraying of diseased plants with gibberellin overcomes some of the symptoms (Agrios, 1988).

Ethylene is another important growth regulator that is induced and produced in fungal-pathogen-infected plants (Cessna et al., 2000; Hamer and Holden, 1997; Mur et al., 2006). Ethylene production in infected tissues can be dramatically induced. This induction is largely dependent on activation of the ethylene biosynthetic pathway in plant tissues. Genes encoding several key enzymes involved in the ethylene biosynthesis are highly activated at the transcriptional level (Cessna et al., 2000; Hamer and Holden, 1997; Mur et al., 2006). Ethylene has been considered as a signal in plants for wounding and senescent responses. Recent studies show that ethylene, together with another signal component jasmonic acid, may play an essential role in plant defense responses of several pathosystems (Mur et al., 2006). In addition to the chemicals described above, fungal pathogens use other chemical weapons in their pathogenesis including polysaccharides, plant defense suppressors, transporters, etc.

## 31.7 STRESSFUL EFFECTS OF FUNGAL PATHOGENS ON HOST PLANTS

### 31.7.1 EFFECTS ON PHOTOSYNTHESIS

Photosynthesis is defined as a function that enables green plants to convert light energy to chemical energy. Plants then use this chemical energy in their processes. In photosynthesis, carbon dioxide taken from the atmosphere and water taken from the soil come together in the chloroplast of the plant cells and with the aid of light energy react and form glucose and release oxygen (Ellis et al., 1981; Wise et al., 2004). As a very important and basic function of green plants, any disruption or interference in photosynthesis can create and induce stressful conditions in plants. Fungal pathogens are among the most important biotic agents that can infect plants, disrupt the photosynthesis process, and induce serious stresses in plants (Agrios, 1988). A very obvious example of the interference of fungal pathogens with the photosynthesis is the chlorosis and necrosis that they cause on the green parts of plants, which then result in reduced growth of many infected plants (Agrios, 1988).

In some diseases caused by fungal pathogens, such as blights or leaf spots, plant photosynthesis is significantly reduced due to the lessening of leaf surface area that is the main photosynthetic part of plants (Rollins, 2003). Photosynthesis in other fungal diseases is also reduced because of the impact of the disease on infected plant tissues and cells. It has been shown that in some fungal diseases, toxins that are produced by these fungal pathogens cause serious inhibition in the production of plant enzymes that are directly or indirectly involved in the photosynthesis (Agrios, 1988). In wilt diseases that are caused by vascular plant fungal pathogens, the stomata are partially closed, chlorophyll is reduced, and photosynthesis is stopped even before wilting symptoms are observed (Agrios, 1988; Van Kan, 2006). All the above-mentioned conditions and negative impacts of fungal plant pathogens on the photosynthesis result in serious pathological conditions in the infected and diseased plants.

### 31.7.2 EFFECTS ON RESPIRATION

The most evident effect of fungal diseases on infected plant respiration is the general increase of respiration as a result of the faster use of reserved carbohydrates by plant tissues (Agrios, 1988). An increase in the respiration usually begins shortly after pathogen attack and continues during multiplication and infection stages. When resistant plants are attacked by fungal pathogens, their respiration increases rapidly compared to susceptible ones, because in resistant plants, higher levels of energy are needed to activate the mechanism of resistance to the pathogen (Agrios, 1988). It has been shown that in infected plants, the activity and concentration of some enzymes involved in the respiration are significantly increased. In infected plants, the accumulation of phenolic compounds that are involved in respiration seems to be higher than healthy plants (Agrios, 1988).

The increase of respiration in plants infected by fungal pathogens can also be explained by an increase in plant metabolism. Studies have indicated that when a plant is infected by a fungal pathogen, its growth is stimulated at first, protoplasmic streaming is increased, and materials are synthesized, translocated, and accumulated in infected tissues (Van Kan, 2006). The energy required for these activities derives from ATP produced during respiration. Since part of this energy is wasted during pathogen infection, an increased respiration is therefore induced to enable the plant to carry out its normal metabolism and activity.

Infection of plants by fungal pathogens also results in the activation of the pentose pathway, compared to those of healthy ones (Palmer et al., 2004). Since the pentose pathway is not directly linked to ATP production, the increased respiration through this pathway fails to produce utilizable energy and is therefore a less efficient source of energy in fungal pathogen-infected plants. As discussed above, an alteration in the normal respiration of plants is also another stressful condition created in plants by fungal pathogens.

### 31.7.3 EFFECTS ON WATER AND NUTRIENT TRANSLOCATION

Most of the plant pathogenic fungi negatively affect the translocation and the movement of water and nutrients in their host plants (Mace et al., 1981). Some fungal pathogens infect plant root and cause a reduced water uptake by the root cells, while many others may grow in the xylem vessels, which results in the blockage of water pathway (Mace et al., 1981). Increased and excessive respiration caused by some fungal pathogens may also create a stressful condition in plants and cause reduced water uptake and movement through the plant system (Mace et al., 1981). It is thought that many plant pathogenic fungi such as damping-off causal agents and the root-rotting fungi cause serious destructions in root tissues before appearance of above-ground symptoms (Mace et al., 1981). Root injuries directly affect the functioning of root cells in the absorption and uptake of water. Some vascular pathogens may affect and reduce water absorption by decreasing root hair production.

The causal agents of fungal damping-off, stem rot, and canker diseases may reach the xylem vessels in the infected area and cause xylem tissue destruction in young plants (Mace et al., 1981). Affected vessels may also be filled with the pathogen structures and with substances produced by the pathogen or by the materials produced by the plant host in response to pathogen infection. The most typical and destructive damage to the xylem system in translocating water has been observed with some wilt-causing pathogens including *Fusarium* (Mace et al., 1981). These pathogenic fungi invade the root xylems and cause diseases primarily by interfering with upward movement of water through the xylem vessels.

In addition to interference with water uptake, fungal pathogens may also affect and damage the plant nutrient translocation system seriously (Mace et al., 1981). Movement and translocation of nutrients produced in leaf cells through photosynthesis occurs through plasmodesmata into adjoining phloem elements and then they move to non-photosynthetic plant cells. Plant pathogens including fungi can potentially interfere with these processes and create stressful conditions in plants.

It is believed that obligate fungal pathogens such as rust and powdery mildew causal agents, can cause accumulation of photosynthetic products and inorganic nutrients in the infected tissues of the host (Mace et al., 1981). Plant pathogens may infect phloem tissues and interfere with the movement of organic nutrients from the leaf cells to phloem or with translocation through phloem elements and possibly with their movement from the phloem into the cells that need to utilize them. However, the synthesis of starch and dry weight are temporarily increased due to the movement and translocation of organic nutrients from uninfected areas of the leaves or from healthy leaves to the diseased tissues and parts of the host plants.

#### **31.7.4 EFFECTS ON CELL MEMBRANE PERMEABILITY**

Plant cell membrane, consists of a double layer of lipid molecules and functions as a permeability barrier that allows passage into a cell only of substances needed by the cell and inhibits passage out of them (Agrios, 1988). Small water-soluble molecules such as ions, sugars, and amino acids flow through or are pumped through special membrane channels made of proteins. In plant cells, only small molecules reach the membrane because of their cell wall. Disruption or disturbance of cell membrane by chemicals or biological factors such as fungal pathogens usually increases permeability of cell membrane (Agrios, 1988; Van Kan, 2006). This results in the loss of useful substances by flowing out and excessive inflow of any substance.

When a fungal pathogen attacks and infects its host plant, alteration in the permeability of plant cell membrane is the first detectable response of the plant to the pathogen invasion. The loss of electrolytes, which are small water-soluble molecules, is the most common result of alteration in cell membrane permeability (Agrios, 1988). Enzymes and toxins produced by fungal pathogens are the most important weapons of pathogens, which are used in this phenomenon. As a result of cell membrane permeability, essential and useful nutrients required for plant growth and reproduction are lost and this creates a stressful condition in plants, which most of the time causes serious damages to plant health and productivity.

#### **31.7.5 EFFECTS ON TRANSCRIPTION AND TRANSLATION**

Transcription and translation are two important processes in plants and play very important roles in plant biology and metabolism (Agrios, 1988, Manners and Scott, 1983; Rollins, 2003). Proteins are made and produced by translation of messenger RNA that is made by transcription of plant cellular DNA. Any disturbance or interference with these processes results in serious damages to normal plant life and induces stressful conditions in plant environment. Fungal plant pathogens, particularly those that are obligate parasites such as powdery mildew and rust causal agents, can potentially affect and interfere with the transcription process in their host plants (Agrios, 1988).

In some fungal infections, the composition, structure, and function of the chromatin associated with the cell DNA are changed by pathogen-induced irritations and this can affect the transcription process significantly. It has been shown that in some plants infected by fungal pathogens, especially in resistant ones, higher levels of RNA are observed compared to the healthy plants (Agrios, 1988). This is particularly true in the early stages of infection. The higher levels of RNA present in infected plants, therefore, cause an increase in transcription rate and the substances synthesized by plant cells.

In addition to transcription, fungal plant pathogens may also affect the translation of messenger RNA to proteins (Manners and Scott, 1983; Van Kan, 2006). Fungal infection usually results in an increase in the level and production of plant enzymes, particularly those involved in the respiration or oxidation and production of phenolic compounds (Agrios, 1988). In addition to this, and as was described before, the level of RNA produced by plant cells are also increased after a plant is infected by fungal pathogens. The increased levels of RNA causes an additional synthesis of proteins through the translation process. An increased production of proteins by plant cells is reflected

by additional levels of enzymes and creates an unusual situation in plant structure and function, which may induce serious stresses in the fungal pathogen-infected plants (Agrios, 1988).

### 31.8 SUMMARY

Fungal plant pathogens attack plants and cause very serious losses to agricultural and horticultural crops. Plant pathogenic fungi and the infection and diseases they cause in the host plants can result in the induction of serious stresses in their hosts. They may affect physiological functions of plants and cause serious damages and disruptions in these functions.

Penetration of fungi into the tissue of their host has been well researched. Pathogenic fungi are facilitated with some mechanical structures such as haustorium and appressorium that can be used in the penetration of fungus into the host plant. In addition to mechanical penetration of host plants, enzymes are also used as biochemical weapons in fungal penetration. Enzymes are subsequently used to continue penetration. Such penetration is achieved by the formation of several groups of enzymes that degrade plant cell walls and enable fungal pathogens to enter their host plant tissues.

After penetration of the fungal pathogens into the host, disease-symptom induction takes place by means of toxins and growth regulators. Fungal toxins may be either non-host specific or host specific. Non-host-specific toxins are produced by phytopathogenic fungi not only in the host plant but also in other species of plants that are not normally attacked by the pathogen in nature. Pathogenic fungi produce host-specific-toxins only when they infect certain hosts.

Growth regulators, which are chemical compounds that occur naturally in plants and act in very low concentrations are also very important in fungal pathogenesis. After fungal pathogens attack and infect plants, the production and occurrence of growth regulators are usually changed and altered and create stressful conditions in plants and crops. Auxin is one of the most important growth regulators. Studies have shown that auxin levels are changed in many diseased plant tissues. Several fungal plant diseases such as corn smut (*Ustilago maydis*) and clubroot of cabbage (*Plasmodiophora brassicae*) show a typical gall symptom due to the increased levels of auxin. Gibberellin is another plant growth regulator produced in some plants. In addition to auxin and gibberellin, ethylene is also produced as a growth regulator in plants. Ethylene has been considered as a signal in plants for wounding and senescent responses.

Plant pathogenic fungi and their penetration, infection, and pathogenesis in plants affect basic plant functions and physiology and induce and create serious stressful conditions. Photosynthesis is the first plant function affected by pathogenic fungi. Fungal pathogens are among the most important agents that can disrupt photosynthesis and induce serious stresses in the plants. A very obvious example of the interference of fungal pathogens in the photosynthesis is the chlorosis and necrosis that they cause on the green parts of plants, which results in reduced growth of many infected plants. In addition to photosynthesis, the respiration of plants is also affected by fungal pathogens. The most evident effect of fungal diseases on infected plant respiration is the general increase of respiration as a result of faster use of reserved carbohydrates by plant tissues.

Plant pathogenic fungi also negatively affect the translocation and the movement of water and nutrients in their host plants. Some fungal pathogens infect the plant root and cause a reduced water uptake by the root cells, while many others may grow in the xylem vessels, which results in the blockage of the water pathway. It is thought that many plant pathogenic fungi such as damping-off causal agents and the root-rotting fungi cause serious destructions in root tissues before appearance of above-ground symptoms. In addition to interference with water uptake, fungal pathogens may also affect and damage the plant nutrient translocation system seriously. It is believed that obligate fungal pathogens such as rust and powdery mildew causal agents, can cause accumulation of photosynthetic products and inorganic nutrients in the infected tissues of the host plant. Plant pathogens may infect phloem tissues and interfere with the movement of organic nutrients from the leaf cells to phloem or with translocation through phloem elements and possibly with their movement from the phloem into the cells that need to utilize them.



Plant cell membrane consists of a double layer of lipid molecules and functions as a permeability barrier that allows passage into a cell only of substances that are needed by cell and inhibit passage out of them. Disruption or disturbance of cell membrane by chemicals or biological factors such as fungal pathogens usually increases permeability of cell membrane. This results in the loss of useful substances by flowing out and excessive inflow of any substances.

Transcription and translation are two major processes in plants and play very important roles in plant biology and metabolism. Proteins are made and produced by translation of messenger RNA that is made by transcription of plant cellular DNA. Any disturbance or interference with these processes results in serious damages to plant normal life and induces stressful conditions in plant environment. In addition to transcription, fungal plant pathogens may also affect the translation of messenger RNA to proteins. Fungal infection usually results in an increase in the level and production of plant enzymes, particularly those involved in the respiration or oxidation and production of phenolic compounds. Additional levels of enzymes create an unusual situation in plant structure and function that may induce serious stresses in the fungal pathogen-infected plants.

As was discussed in this review, plant pathogenic fungi are among the most important biotic agents that can seriously affect plant and crop health and cause serious damages and injuries to different parts of plants during various stages of their life cycle. These injuries and damages can result in the induction of stressful conditions in plants that make plants to suffer and even die due to the diseases and disorders caused by fungal pathogens. In order to have healthy and productive plants and crops, it is extremely important to protect them against fungal pathogens as was reported by several workers cited in this chapter. This can be done by using a combination of different strategies including chemical, cultural, biological, and genetic control methods to combat and overcome fungal plant diseases as was reported by several workers cited in this chapter. The successful application and implementation of control strategies will hopefully reduce damages and stresses caused and induced by fungal plant pathogens and will be a promising approach to a sustainable agriculture.

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# *Part VI*

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## *Genetic Factors and Plant/Crop Genomics under Stress*

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# 32 Genetic Factors Affecting Abiotic Stress Tolerance in Crops

*Arun Kumar Joshi*

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32.1 INTRODUCTION

Plant growth and development is a result of the interplay between the genetically governed potential of the plant and the plant environment in which it grows (Lewis 1976). Therefore, plant performance is often affected by a number of unfavorable environmental factors, among which abiotic factors are of crucial importance. Since plants are immobile, they suffer more compared to other organisms. The significance of abiotic stresses can be understood from the fact that the earth’s surface, which is 70% salt water and 30% land, possesses only half of its land area free from extremes of water and temperature, and to soil erosions or difficult geography (Lewis and Christiansen 1981). The other half that is used for agricultural production has to face a number of abiotic stresses that are considered to be the main source of yield reductions all over the world (Boyer 1982, Cushman and Bohnert 2000). The recent reports of climate change (IPCC 2007, Battisti and Naylor 2009) are expected to further aggravate the threat of abiotic stresses.

Abiotic stresses are generally of two types: stable and fluctuating. Stable stresses are caused by abnormal pH or metal toxicity, whereas unstable or fluctuating stresses include abnormal levels of water (drought/flooding), temperature (cold/hot), and other factors such as pollutants. Abiotic stresses are characterized by the occurrence of more than one stress at a particular growth stage or throughout the growing cycle, even though one stress may dominate. Since the whole biotic world, directly or indirectly, is dependent on plants for survival, any stress to plants gets reflected in their own stress. This is of utmost importance for human beings and the present-day agriculture when our major natural resources are shrinking except the number of mouths to feed. Therefore, providing relief to crop plants from abiotic stresses is providing relief to man himself. Relief from abiotic stresses is possible either by changing/avoiding the environment or changing the genotype of the plant itself. Man has tried to improve stress tolerance of plants from the day he started crop domestication and cultivation. However, probably abiotic stress tolerance was not as important for food security as it is today. The improvement in the abiotic stresses tolerance of crops was rather slow for a long time due to lack of knowledge about their genetic control. With the advancement of knowledge about the genetic factors affecting abiotic stress tolerance, there is a new hope for developing significantly better stress tolerance crops.

Breeding for abiotic tolerance may be direct (selection pressure under stress) or indirect (selection pressure under stress-free environment) (Lewis and Christiansen 1981). The simplest approach to breeding for stress tolerance is to select for yield, which is the integrating trait, and to carry out the selection in a representative stress environment. It can be enhanced by carefully managed stresses (Bänziger et al. 2006, Lafitte et al. 2006) and by intelligent choice of parents to pyramid desirable traits (Yeo and Flowers 1986). The approach of using fewer crosses with larger population (Witcombe and Virk 2001) can be very effective, which has been proved in breeding of stress-tolerant, widely adapted rice (Joshi et al. 2007a–e).

Recently, molecular tools, especially molecular markers, have been added in this direction. The advent of molecular markers has revolutionized the genetic analysis of crop plants and provided not only geneticists, but also physiologists, agronomists, and breeders with valuable new tools to identify traits of importance in improving resistance to abiotic stresses (Rudd et al. 2005), and is seen as an approach for precision plant breeding in the twenty-first century (Collard and Mackill 2008).

Marker-assisted selection can be effective in enhancing efficiency, but, at least so far, the selection for markers linked to component traits of low heritability has not produced predicted outcomes (Witcombe et al. 2008). Fine-mapping and map-based cloning of quantitative trait loci

(QTLs) (Price 2006) will allow them to be used more effectively in breeding by eliminating the effects of unwanted linked alleles. Marker Assisted Selection (MAS) need to be fine-tuned, so that precise combinations of alleles can be combined for maximum effect. Marker-assisted breeding is now increasingly targeted toward tracking the candidate genes responsible for stress tolerance through gene identification and functional studies (Tuberosa and Salvi 2004, Mantri et al. 2007). Till date, the expression profiles of all the genes in genome have been investigated in some of the model crops, such as *Arabidopsis thaliana* (thale cress), *Oryza sativa* (rice), *Medicago truncatula* (barrel medic), and *Populus trichocarpa* (black cottonwood). Other crops where this work has been possible are *Brachypodium distachyon*, *Lotus japonicus* (lotus), *Manihot esculenta* (cassava), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Sorghum bicolor*, *Zea mays* (corn), and soybean (<http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html>). Transcription factors (TFs) that bind to DNA through specific *cis*-regulatory sequences and either activate or repress gene transcription have been reported to act as control switches in stress signaling (Tran and Mochida 2010). The recent completion of the soybean genomic sequence has open wide opportunities for large-scale identification and annotations of regulatory TFs in soybean for functional studies. Transgenic and functional genomics approaches hold tremendous promise for the future and are being pursued vigorously to improve qualitative and quantitative traits, including tolerance to biotic and abiotic stresses in different crops (Ashraf 2010).

## 32.2 DROUGHT TOLERANCE

The ability of a crop to grow satisfactorily in areas subjected to water deficits has been termed drought resistance (Turner 1986). Crops all over the world are exposed to chronic or sporadic periods of drought (Boyer 1982), a multidimensional stress affecting plants at various levels of organization (Blum 1996). Drought stress affects the yield by reducing both sink and source. It can be a result of stress affecting either one of these directly or their interaction with one another (Blum 1996). Before seeing the external world, the living plant (the dormant embryo) within the seed is highly tolerant to desiccation, but loses its tolerance upon germination and emergence. It has been suggested that a unified abiotic stress resistance mechanism for drought at the level of the whole plant or the single gene is probably not present (Blum 2004). Plants survive under drought through the avoidance or postponement of dehydration or tolerance (Turner 1986). The traits associated with avoidance and tolerance can be constitutive (differing between genotypes) or adaptive (varying with the stage of the life cycle) (Witcombe et al. 2008). Drought avoidance and drought tolerance involve different mechanisms and processes, and phenology is the single-most important factor influencing whether a plant avoids drought. In other words, plants tolerate drought stress through various morphological, biochemical, and molecular adjustments at the whole plant level.

### 32.2.1 MORPHOPHYSIOLOGICAL TRAITS: GENETIC BASIS

Though there are no traits that confer global drought tolerance (Passioura 1996), numerous constitutive traits carry a large impact on crop performance under drought stress (Blum 1996). According to Wilson (1981), traits associated with water use efficiency act through their effect on (1) the timing of crop development, (2) the efficiency of root to harvest water, (3) the effective transpiration control by shoots and the relationship of transpiration and photosynthesis, and (4) the ability of plants to endure stress. Any trait that reduces transpiration or increases photosynthesis will increase the water use efficiency. Ludlow and Muchow (1990) have reviewed important traits in crops for success in water-limited environments. Any trait that reduces transpiration or increases photosynthesis will increase the water use efficiency. Though several traits join together to fight drought, the genetics of some of the traits useful under drought conditions is given below.



### 32.2.1.1 Earliness

Rapid plant development and early maturation require less water, and thus work through drought avoidance (Turner 1979, 1986, Ludlow and Muchow 1990, Boyer 1996). There is genetic variation for earliness, both across and within species (Hall and Grantz 1981, Richards and Passioura 1989, Richards 2004). For example, in wheat, there is much genetic variation for flowering time and maturity (Innes and Quarrie 1987). The *Rht* genes in wheat possibly possess a pleiotropic effect on earliness (Innes and Quarrie 1987). In maize, evidence for numerous small-effect QTLs was found to control the flowering time (Buckler et al. 2009); however, no individual QTLs were identified at which allelic effects are determined by the geographic origin or large effects for epistasis or environmental interactions. Thus, in contrast to rice and *Arabidopsis*, a simple additive model was suggested to accurately predict the flowering time for maize (Buckler et al. 2009).

### 32.2.1.2 Roots

An extensive root system is desirable for efficient water extraction in different crops (Hurd 1974, Lorens et al. 1987, Boyer 1996). Genetic variation in root characteristics does exist in crop plants (Hurd 1974, Taylor et al. 1974, Boyer et al. 1980, Lehman and Engelke 1993, Sarker et al. 2005). Many root characteristics have been shown to be under genetic control and are quantitatively inherited. But in rice, the difference in the depth of rooting was controlled by only a few genes (Armenta-Soto et al. 1983, Ekanayke et al. 1985). In wheat, genetic variation for root hydraulic conductance is present and is heritable (Blum and Johnson 1993). Genetic variability for root size was found in sorghum (Blum et al. 1997), wheat (O'Brien 1979), rice (Larsson and Svenningsson 1986), soybean (Boyer et al. 1980), and oat (Murphy and Nelson 1982). The density of root hairs also shows considerable genetic variability (200 cm<sup>2</sup> in trees to 2500 cm<sup>2</sup> in cereals), but little is known about intraspecific variability and the genetics of this trait (Monneveux and Belhassen 1996). Root traits (root length, root number, root-tip thickness, and root/shoot weight ratio) in rice have been found to possess moderate heritability and are under the control of both additive and dominant gene effects (Armenta-Soto 1983, Ekanayke 1985). Some of these traits also showed heterosis in several crop plants (Sinha and Khanna 1975, Blum 1996). The growth angle and the number of seminal roots in wheat are also reported to show a significant genotypic variation (Manschadi et al. 2008).

### 32.2.1.3 Stomatal Conductance

The control of leaf stomatal conductance (g<sub>s</sub>) is a crucial mechanism for plants, since it is essential for both CO<sub>2</sub> acquisition and desiccation prevention (Dodd 2003, Medici et al. 2007). A reduced stomatal conductance through various characteristics, such as stomatal frequency, length, and behavior (Ludlow and Muchow 1990), increases the water use efficiency. Genetic variation is reported for various stomatal characters (Clarke and Townlet-Smith 1984, Jones 1987), and they seem to be highly heritable (Roark and Quisenberry 1977). In addition, dimensions, and especially frequency, can change more than twofold in response to radiation or to water status, or according to developmental stages (Jones 1987, Roark and Quisenberry 1977). Monneveux et al. (2006) reported that durum wheat breeding at maturity would lead, under Mediterranean drought conditions, to higher stomatal conductance, lower transpiration efficiency, and higher grain yield. Medici et al. (2007) reported that in maize, the stomatal conductance of drought-tolerant varieties was lower compared to other cultivars.

### 32.2.1.4 Epicuticular Wax

Many characteristics of the leaf affect drought tolerance, which include cuticular wax characteristics (Cameron et al. 2006). Likewise, a waxy layer covering the plant parts, for example, the glaucous character in wheat and the bloomed trait in sorghum, improves the water use efficiency. Genetic variation is reported for the bloomed trait in sorghum (Ebercon et al. 1977, Jordan et al. 1983) and the glaucousness in wheat (Nizam Uddin and Marshall 1988). Variation for cuticular wax is also reported in other crops, for example, oat (Bengtson et al. 1978) and rice (O'Toole et al. 1979).

The genetics of epicuticular wax has been investigated in several crop species. The presence of waxy bloom was found to be controlled by a single dominant gene in sorghum (Monneveux and Belhassen 1996). However, heritability for the bloomed trait of sorghum was low (Jordan et al. 1983). In tobacco, a sixfold increase of tree tobacco lipid transfer proteins (LTPs) gene transcripts was observed after three drying events, providing further evidence that LTP is involved in cuticle deposition (Cameron et al. 2006). In wheat also, a dominant non-glaucousness gene was reported from synthetic hexaploid wheat (*Triticum aestivum* L.), which has been mapped in chromosome 2DS (Liu et al. 2007).

### 32.2.1.5 Osmotic Adjustment

Osmotic adjustment (OA) reduces the rate of leaf senescence (stay-green trait) because it increases both avoidance and tolerance to dehydration. Genetic variation for this trait has been found in wheat (*ms* gene) (Morgan 1977, 1984, Blum et al. 1983, Morgan and Condon 1986), sorghum (Akerson et al. 1980, Wright and Smith. 1983, Blum and Sullivan 1986), millet (Hensen 1982), cotton (Karami et al. 1980), rice (Lilley et al. 1996), and pigeon pea (Flower and Ludlow 1987). OA is simply inherited and only one or few genes are involved in wheat (Morgan 1977, 1991) and soybean (Basnayake et al. 1995). In rice, the *indica* cultivars tend to be more dehydration tolerant than *japonica* cultivars (Lilley et al. 1992). A gene for OA was located in chromosome 7A of wheat (Morgan 1991). On the basis of homology between a small segment of chromosome 7 of wheat and chromosome 8 of rice (Ahn et al. 1993, Van Deynze et al. 1993), it has been suggested that there might be an association between the OA gene of wheat and rice. Jiang and Huang (2001) demonstrated that drought preconditioning enhanced heat tolerance in Kentucky bluegrass (*Poa pratensis* L.), which could be related to the maintenance of higher OA associated with the accumulation of ion solutes and water-soluble carbohydrates and the development of extensive roots deeper in the soil profile.

In maize, the relationship of OA and yield maintenance under drought was not established for a long time. However, a study (Chimenti et al. 2006) compared two S4 populations derived from a cross between inbred lines exhibiting the highest and lowest capacities for OA in a screening applied to 20 inbred lines. Crops of these populations were grown under a rainout shelter and subjected to 30-day droughts either before or during flowering. They concluded that OA can contribute to drought tolerance in maize crops exposed to water deficit both before and during flowering, and that the trait carries no yield penalty under irrigation.

### 32.2.1.6 Transpiration Efficiency

The transpiration efficiency of  $C_4$  plants is greater than  $C_3$  plants (El-Sharkawy 2009). Genetic variation for transpiration efficiency has been reported in wheat, barley, cotton, peanut, and sunflower (Blum and Johnson 1993). Its inheritance is complex (Ludlow and Muchow 1990) but heritability is high (Martin and Thorstenson 1988). High heritability has been noted in crested wheat grass (Johnson et al. 1990), peanut (Hubic et al. 1988), and wheat (Ehdaie et al. 1991), and moderate heritability in cowpea (Hall et al. 1993).

Several other traits, such as mobilization of preanthesis assimilates, leaf movements, epidermal conductance, developmental plasticity, and leaf area maintenance, are also important for drought tolerance (Ludlow and Muchow 1990). Genetic variation for some of these traits is also reported, for example, for the mobilization of preanthesis assimilates (Blum et al. 1983, Constable and Hearn 1978) and leaf area maintenance (Duncan et al. 1981, Rosenow et al. 1983). Richards and Passioura (1981) found intraspecific variability in bread wheat for the xylem vessel diameter, which possesses high heritability. Leaf rolling showed genetic variation in sorghum (Chang et al. 1974, Beg 1980) and rice (Chang et al. 1974, Turner 1986). Leaf rolling was also found to have an association with drought tolerance in rice (Efisue et al. 2009). Genetic variation for epidermal conductance has been observed in rice (Yoshida and Reyes 1976), sorghum (Jordan et al. 1984, Blum 1979) and soybean (Manavalan et al. 2009).

### 32.2.2 GENETICS OF DROUGHT TOLERANCE

Genetics of drought resistance can be partly understood through the inheritance of traits responsible for drought avoidance or postponement or tolerance, as described above. However, the situation is complex as single genes that substantially change water use efficiency are difficult to find and it generally involves many genes and many interactions (Boyer 1996). Tolerance to drought is rare in vegetative parts of plants, while angiospermous seeds and pollen are able to survive extreme dehydration (Leopold et al. 1992). Genes responsive to drought, desiccation, high osmoticum, or wilting have been identified in tomato (Cohen and Bray 1990), *Craterostigma plantagineum* (Bartels et al. 1990, Bartels and Salamini 2001, Bartels 2005), maize (Close et al. 1989, Tuberosa et al. 2002), barley (Close et al. 1989), rice (Mundy and Chua 1988), *Arabidopsis* (Gilmour et al. 1992), tobacco (La Rosa et al. 1989), soybean (Surowy and Boyer 1991), cotton (Baker et al. 1988), and wheat (Kirigwi et al. 2007, Gupta et al. 2009).

The location of genes having a major effect on drought-induced abscisic acid (ABA) accumulation in wheat was determined by using molecular markers, a set of single chromosome substitution lines and populations derived from a cross between high and low ABA-producing genotypes (Quarrie et al. 1994). A similar drought test with detached and partially dehydrated leaves confirmed the location of gene(s) regulating ABA accumulation in the long arm of chromosome 5A (Quarrie et al. 1994). The MAPMAKER QTL showed the most likely position for the ABA QTL to be between the loci *Xpsr575* and *Xpsr426*, about 8 cm from *Xpsr 426*; another QTL for ABA accumulation may be present on chromosomes 3BS and 6AL (Quarrie et al. 1994).

QTL for response to drought has been reported in different crops, such as maize (Paterson et al. 1991, Lebreton et al. 1995, Chenu et al. 2009), sorghum (Tuinstra et al. 1998, Crasta et al. 1999, Sanchez et al. 2002), rice (Kato et al. 2008, Bernier et al. 2009), wheat (Quarrie et al. 1994, Kirigwi et al. 2007, Matthews et al. 2008, Gupta et al. 2009), and barley (Sanguineti et al. 1994). There is clear evidence now that all the major cereal species have extensive linkage blocks where the gene order is conserved (Moore et al. 1995).

QTLs have been identified for several drought resistance component traits in rice (McCouch 1995, Lilley et al. 1996, Price and Tomos 1997, Yadav et al. 1997, Ali et al. 2000, Price et al. 2000, Tripathy et al. 2000, Zheng et al. 2000, Zhang et al. 2001, Li et al. 2005, Gomez et al. 2006, Kato et al. 2008). In one of such studies (Lilley et al. 1996) to understand the genetic mechanism of OA and dehydration tolerance in rice, a recombinant inbred ( $F_7$ ) population was mapped with 127 restriction fragment length polymorphism (RFLP) markers; a major locus was found to be associated with OA in rice (Lilley et al. 1996). This locus may be homologous with a single recessive gene previously identified for the same trait in wheat; the putative OA locus and two of five QTLs associated with the dehydration tolerance were close to the chromosome regions associated with root morphology (Lilley et al. 1996). Specht et al. (2001), using molecular marker analyses of the 236 RILs (recombinant inbred line), reported the detection of three QTLs in soybean with very strong effects on the transpiration efficiency associated with drought. In sorghum, Sanchez et al. (2002) identified four genomic regions associated with the stay-green trait using an RIL population developed from B35  $\times$  T  $\times$  7000. These four major stay-green QTLs were consistently identified in all field trials and accounted for 53.5% of the phenotypic variance. Likewise, QTLs linked to morphophysiological and plant production traits in rice under drought stress in the field were mapped by evaluating 177 F6 recombinant inbred lines of Bala  $\times$  Azucena under rainfed (Gomez et al. 2006). A total of 24 QTLs were identified for various traits under stress, which individually explained 4.6%–22.3% of phenotypic variation. Composite interval mapping detected three markers, namely, RM3894, RG409, and G1073, on chromosomes 3 and 8 linked to grain yield under drought stress in temperature extreme (TE), respectively, explaining 22.3%, 17.1%, and 10.9% of phenotypic variation (Gomez et al. 2006). QTLs for leaf drying, days to 50% flowering, and number of productive tillers under drought stress collocated at certain of these regions. Further, QTLs for several root traits overlapped with QTLs for grain yield under stress in these recombinant inbred (RI) lines, indicating the pleiotropic effects of root trait QTLs on rice performance under stress (Gomez et al. 2006).

Kato et al. (2008) reported three QTLs on chromosomes 2, 4, and 7 for the relative growth rate of rice. The QTL on chromosome 7 had a constant effect across environments, while the QTL on chromosome 4 had an effect only under nonstressed conditions, and that on chromosome 2 only under stressed conditions. The stress-specific QTL on chromosome 2 was not colocated with any QTLs for the root system depth previously reported from the same mapping population. However, this QTL was colocated with a stress-specific QTL for specific water use (SWU), suggesting that the control of transpiration was relevant to dry matter production under drought. Recently, Bernier et al. (2009) reported a large-effect QTL (*qtl12.1*) for grain yield under drought conditions tested across 21 field trials: 10 at IRRI (International Rice Research Institute) in the Philippines and 11 in eastern India. The relative effect of the QTL on grain yield increased with the increasing intensity of drought stress, from having no effect under well-watered conditions to having an additive effect of more than 40% of the trial mean in the most severe stress treatments. The QTL improved grain yield in 9 out of 10 direct-seeded upland trials where drought stress was severe or moderate, but no effect was measured under well-watered aerobic conditions or under transplanted lowland conditions. This confirmed that *qtl12.1* has a large and consistent effect on the grain yield under upland drought stress conditions, in a wide range of environments (Bernier et al. 2009).

Chenu et al. (2009) proposed a modeling approach to bridge the “gene-to-phenotype” gap caused by the genotype  $\times$  environment interaction in maize under drought. They simulated the impact of QTLs controlling two key processes (leaf and silk elongation) that influence crop growth, water use, and grain yield. The simulations obtained illustrated the difficulty of interpreting the genetic control of yield for genotypes influenced only by the additive effects of QTLs associated with leaf and silk growth (Chenu et al. 2009).

### 32.2.3 GENE EXPRESSION

Water deficit is one of the most common abiotic stresses that affects the growth and development of plants through alterations in metabolism and gene expression (Leopold 1990). The molecular basis of dehydration stress responses in cells and organisms has been intensively researched over the past years (Somvanshi 2009). Molecular and genomic analyses have facilitated new gene discovery and enabled genetic engineering to deploy several functional or regulatory genes to activate specific or broad pathways related to drought tolerance in plants (Umezawa 2006).

Genetic information to withstand drought is present in plants in stress genes, but these genes are expressed only in particular developmental stages (Mckersie and Leshem 1994). The molecular studies of dehydration stress are mainly based on (1) the desiccation tolerance of the maturing embryo (Quatrano 1989) and resurrection plants (angiosperms that are able to survive dehydration and revive upon hydration) (Bartels 1990, Gaff 1971), (2) *A. thaliana* (Yamaguchi-Shinozaki 1993, Kasuga et al. 1993), and (3) other crops such as tomato, pea, wheat, and barley (Bartels et al. 1996). Genes regulated by drought stress can be divided into three groups (Bartels et al. 1996): genes encoding polypeptides of unknown functions, genes encoding *Lea* proteins and related polypeptides, and genes encoding polypeptides of known functions.

The DNA sequence analysis of osmotic stress-inducible cDNAs indicates that genes responsible to drought encode a variety of proteins (Wood and Goldsbrough 1997). Many of the proteins encoded by these cDNAs have been classified into various groups, namely, LEA (late embryogenesis abundant) (Baker et al. 1988), RAB (responsive to ABA) (Skriver and Mundy 1990), and dehydrin (Close et al. 1989) proteins. LEA proteins and dehydrins are classified on the basis of their characteristic amino acid motifs, while RAB proteins are classified based upon the expression in response to ABA (Wood and Goldsbrough 1997). LEA proteins, first identified during seed maturation and desiccation, express in water-stressed vegetative tissues in almost all plants (Wood and Goldsbrough 1997). They are supposed to protect the dehydrating cells by a variety of mechanisms, including renaturation of unfolded proteins, sequestration of ions, and stabilization of the native protein structure (Wood and Goldsbrough 1997). Recently, it has been demonstrated that the

accumulation of the barley HVA1 protein, a group of three LEA proteins, in transgenic rice confers an increased tolerance to water deficit as well as to salt stress (Xu et al. 1996).

Genes have also been isolated from the resurrection plant *C. plantagineum*, which can recover completely from complete dryness within 24 h of contact with water (Schneider et al. 2004). Many of the desiccation-induced genes share sequence homology with LEA genes (Galau et al. 1986, Piatkowski et al. 1990). ERD genes (early responsive to dehydration stress genes) in comparison to ABA-responsive genes are preferentially responsive to the dehydration stress (Jensen et al. 1996).

The dynamics of water transport in plants is influenced by water channel proteins in plants (Verkman 1992, Chrispeels and Maurel 1994), which are related to the super family of membrane-intrinsic proteins (MIP) first characterized in *Escherichia coli*. The functions of different MIP channels vary (Reizer et al. 1993). Several MIP-related proteins have been identified in plants. A member of this family, *Trg 31* (in pea), was initially identified when the gene expression was induced in partially dehydrated leaves (Guerrero et al. 1990). Another dehydration-inducible gene was isolated from *A. thaliana* (Yamaguchi-Shinozaki 1992). NOD 26, the first plant MIP protein to be characterized (Miao et al. 1992), is abundant in the prebacteroid membrane root nodules. MIP-like tonoplast intrinsic proteins (TIPS) and their corresponding genes have been identified (Chrispeels and Maurel 1994).

Water deficit results in diminished growth of young leaves. In soybean, water-stressed plants show an increased expression of genes encoding the vegetative storage proteins (*Vsp*) (Mason and Mullet 1990). But the same does not occur in mature leaves even though they possess the potential to express *Vsps* when leaves are wounded (Mullet and Whitsitt 1996). In a study (Rizhsky et al. 2002), the combination of drought and heat shock resulted in the closure of stomata, suppression of photosynthesis, enhancement of respiration, and increased leaf temperature. Some transcripts induced during drought, for example, those encoding dehydrin, catalase, and glycolate oxidase, and some transcripts induced during heat shock, for example, thioredoxin peroxidase and ascorbate peroxidase, were suppressed during a combination of drought and heat shock. In contrast, the expression of other transcripts, including alternative oxidase, glutathione peroxidase, phenylalanine ammonia lyase, pathogenesis-related proteins, a WRKY TF, and an ethylene response transcriptional coactivator, was specifically induced during a combination of drought and heat shock (Rizhsky et al. 2002). Photosynthetic genes were suppressed, whereas transcripts encoding some glycolysis and pentose phosphate pathway enzymes were induced, suggesting the utilization of sugars through these pathways during stress (Rizhsky et al. 2002). In chickpea, transcriptional profiling indicated that genes are differentially regulated in response to drought tolerance (Mantri et al. 2007), although the same behavior was displayed under high-salinity and cold stresses (Mantri et al. 2007).

Transgenic breeding is also being viewed as a science of the future for drought tolerance. In a recent study (Wang 2009), using *Agrobacterium*-mediated transformation, a wheat LEA gene, TaLEA3, was integrated into *Leymus chinensis* (an important grassland perennial grass). The transgenic lines showed an enhanced growth ability under drought stress, during which transgenic lines had increased the relative water content, the leaf water potential, and the relative average growth rate, but decreased the malondialdehyde content compared with non-transgenic plants (Wang 2009). A large number of drought-tolerant genes are being patented around the world. It has been reported (Somvanshi 2009) that almost all of the patented dehydration stress tolerance genes from different organisms were used in engineering drought tolerance in crop plants. Several lines of evidence have indicated that molecular tailoring of genes has the potential to overcome a number of limitations in creating drought-tolerant transgenic plants (Umezawa 2006). This suggests that in future, the transgenic approach would be playing a much greater role in drought tolerance than is visible today. However, the evaluation of the transgenic plants under stress conditions, and understanding the physiological effect of the inserted genes at the whole plant level remain the major challenges to overcome (Bhatnagar-Mathur et al. 2008).

### 32.2.4 BREEDING

The understanding of the genetic basis of drought tolerance is poor (Ortiz et al. 2008a). Despite many decades of research, drought continues to be a major challenge to agricultural scientists, probably due to the difficult nature of the target environment and the interaction of drought with other abiotic as well as biotic stresses (Blum 1988). This is supported by the observation that in water-limited environments, the yield of the biomass of current cultivars is about the same as that of cultivars from over a century ago (Siddique et al. 1989). However, the wondrous display of plant adaptations to dry habitats points to the fact that a substantial genetic variation for drought tolerance exists, and this may be used for plant breeding (Richards 1996).

The problem of breeding crops for drought environments is not due to the want of enough genetic variation, but probably lies in the elusive design of the ideal plant/the ideotype (Donald 1968), which has been in attention (Sedgley 1991) for both normal and stress conditions (Blum 1996). The demand of genes depends on the type of ideotype required for the water stress area. Since a large number of traits take part in plants' stress response, the task is not easy in practical terms. The water status of a plant is a function of uptake (by roots) and loss (via stomata and cuticle) of water; therefore, breeding strategies may broadly focus on either of these two parameters (Kuckuck et al. 1991). So far, the breeding strategies for drought areas have been suggested to depend on (1) selecting genotypes with an improved yield in water stress environments (Mullet and Whitsitt 1996), (2) identifying and selecting traits that contribute to drought avoidance, drought tolerance, or water use efficiency (Ludlow and Muchow 1990), and (3) even selecting genotypes under nonstress environments and then trying in stress areas (Sayre et al. 1995, Richards 1996). Drought stress is highly variable in its timing, duration, and severity, and therefore displays high environmental variation and  $G \times E$  variation (Witcombe et al. 2008). The use of managed stress environments can be very effective in breeding for drought tolerance; however, it is important to apply a sufficient drought stress intensity to maximize  $G \times E$  (Bänziger et al. 2006). In other words, without plant selection under water deficient conditions, traits beneficial under water stress may be missed (Boyer 1996). However, in favorable environments, there is less error, and thus, a high yield potential expressed in favorable environments can also have a spin-off in less favorable environments (Richards 1996). In Australia, where wheat is grown in a water-limited environment, 95% of current cultivars can be traced to the CIMMYT germplasm where breeding is done under highly favorable environments (Richards 1996).

In wheat, considerable progress has been made in yield improvement under drought in recent decades using the available gene pool and selecting under drought stress (Trethowan et al. 2002). Opportunities exist to improve the tolerance further, if new genetic variability can be combined with existing variability and if the underlying genetic control of tolerance can be better understood (Ortiz et al. 2008b). The resynthesized wheat lines developed by crossing the modern durum wheat with *Aegilops tauschii*, the probable donor of the D genome in hexaploid wheat, have introduced new genetic variation into the wheat gene pool for many characters. Not surprisingly, resynthesized wheat lines have also been a source of variation for drought and heat tolerance (Trethowan et al. 2002). Some advanced materials derived from resynthesized wheat lines have improved adaptation worldwide, especially in drought-stressed environments.

Recognizing water productivity and water use efficiency as priorities for wheat, CIMMYT researchers disaggregated grain yield under water stress into distinct components to apply these findings to the genetic enhancement of this crop (Reynolds and Borlaug 2006). Ongoing research is providing a better understanding of traits with major effects on the water productivity in dryland wheat areas (Reynolds et al. 2007). These include root architecture and physiological traits, resistance to soil-borne pests and diseases, tolerance to heat and salinity, and zinc-deficient and boron-toxic soils. The combination of improved germplasm; the Center and partners' expertise in drought physiology, soil-borne diseases, and agronomy; and the availability of DNA markers for various traits place CIMMYT in a unique position to develop water-productive wheat with resistance to the important stresses for use by partners throughout the developing world (Ortiz et al. 2008b).

Some important attributes for drought-prone environments are available in the wild relatives of wheat (Reynolds et al. 2007). Resynthesizing hexaploid wheat with wild ancestors has been used at CIMMYT for tapping this useful variation and incorporating such genetic resources into wheat-bred germplasm (Dreccer et al. 2007). Recently, Ogbonnaya et al. (2007) found that such lines derived from resynthesizing wheat yielded 8%–30% higher than the best local check in multisite trials across diverse regions of Australia. Their results reinforce previous research conducted at CIMMYT that lines derived from synthetic wheat have the potential to significantly improve grain yield across environments.

QTL mapping techniques are a hope of the future with regard to improved abiotic stress tolerance and drought stress in particular. The QTL locations are now easy to compare across species, as demonstrated through the comparison of QTL of root characters in maize and rice (Lebreton et al. 1995, Buckler et al. 2009). A decade ago, a marker-assisted selection using random-amplified polymorphic DNA (RAPDs) was demonstrated to improve the yield performance in common bean in stress conditions (Schneider et al. 1997). In rice, Li et al. (2005) suggested that the high yield potential and drought tolerance/water use efficiency (DT/WUE) can be combined more efficiently by the designed QTL pyramiding (DQP) strategy. Though molecular genetics might prove important, the identification of stress-responsive genes or even their cloning and insertions seem to be beyond practical application, unless their function and value within the ideotype can be demonstrated (Blum 1996).

There are a few reports of the transfer of alien DNA into crop species specifically to improve drought responses, but an extensive introgression programme is in progress to transfer useful traits from *Festuca* into *Lolium* (Thomas et al. 1994). In some of the introgression lines with *Festuca arundinacea* in *Lolium multiflorum* × *F. arundinacea* crosses, drought resistance was equivalent to the *Festuca* donor (Thomas et al. 1994). This single chromosome addition line of *F. arundinacea* on to *L. multiflorum* also showed improved drought resistance (Quarrie et al. 1997). Hence, this work looks promising in future to improve drought responses of other graminaceous crops (Quarrie et al. 1997). The introgression of the drought-tolerant mechanism present in wild species in cultivated plants is in course in several laboratories. Another strategy for the genetic improvement of plants under drought has been to identify gene(s) of desiccation tolerance, for example, in desert plants or wild species (Bartels and Nelson 1994, Bohnert et al. 1995), and transfer them to agronomic crops (Tardieu 1996). A transgenic rice plant having tolerance to water deficit and osmotic stress has been reported (Nguyen et al. 1997). In wheat, transgenic approaches for incorporating stress-inducible regulatory genes that encode proteins such as TFs (e.g., *DREB1A*) into the wheat cultigens pool are also being pursued (Hoisington and Ortiz 2008).

### 32.3 SUBMERGENCE TOLERANCE

Submergence tolerance is defined as the ability of crop plants to survive and continue growing after being completely submerged in water for several days. Tolerance to water logging or submergence tolerance is associated either with crops grown in high rainfall areas of the world or in lowland areas that are kept flooded by natural rain, flood, or canal irrigation. Most of our knowledge regarding submergence tolerance is obtained from the studies on rice crop, 16% of whose total world area is affected by this problem (Khush 1984). Compared to other parts of the world, flooding is a serious, naturally occurring problem for rice production in the rainfed lowlands of south and southeast Asia during the monsoon season. It is estimated that around 50% of the rice-growing area in this ecosystem is affected by flash flooding at various stages of growth (Dey and Upadhyaya 1996).

Limited gas diffusion in water is considered the principal cause for the adverse effects of submergence (Setter et al. 1995). Therefore, tolerance to flooding is associated to cope up with problems associated with submergence, such as anaerobiosis, lower carbon assimilation due to less CO<sub>2</sub> and radiation (Setter et al. 1987, 1989), and high ethylene. This is partly achieved by avoidance—through maintenance of growth processes leading to the elongation of plants to maintain their foliage above water (Mohanty and Choudhary 1986).

### 32.3.1 MORPHOPHYSIOLOGICAL TRAITS: GENETIC BASIS

Several morphophysiological traits have been reported to be associated with submergence tolerance (Setter et al. 1997). Setter et al. (1997) listed 17 morphophysiological traits as part of the mechanism explaining submergence tolerance in rice. These traits were classified into presubmergence, submergence, and postsubmergence traits. Among these, the three important traits are (1) carbohydrate concentration, (2) alcoholic fermentation, and (3) elongation of the stem. Favorable effects of high carbohydrate concentration (Palada and Vergara 1972, Setter et al. 1987, Mallik et al. 1995) and high alcoholic fermentation (Waters et al. 1991, Ricard et al. 1994) are well documented. Stem elongation does favor through avoidance, but, on the other hand, there is a strong negative correlation between elongation growth and percent survival of seedlings during submergence (Setter et al. 1994), because elongation growth competes for energy and carbohydrates required for maintenance processes for survival (Setter et al. 1997). The elongation mechanism is effective only when the water level remains high for a considerable period, as in deep water rice culture. It is not desirable under flash flood conditions, because when the water recedes, the plants tend to lodge (Seshu and Akbar 1995).

The capacity to survive submergence depends not only on specific environmental factors, but also on the strategy that plants have evolved for adoption to particular flood-prone environments (Ram et al. 2002). For instance, in rice, the two main strategies are to elongate and escape, or not to elongate and conserve resources. For rainfed lowland rice exposed to flash flooding, the elongation growth during complete submergence has major adverse effects on survival, presumably since this competes with maintenance processes that require carbohydrates and energy. Therefore, selection for minimal elongation during submergence is currently being exploited as a trait for submergence tolerance by rainfed lowland rice breeders in south and southeast Asia (Ram et al. 2002).

Recently, *Erythrina speciosa* was shown to display 100% survival until the 60th day of flooding and was able to recover its metabolism (Medina et al. 2009). The recovery during soil flooding appeared to be associated with morphological alterations, such as the development of hypertrophic lenticels, adventitious roots, and aerenchyma tissue, and with the maintenance of neutral amino acids in roots under long-term exposure to root-zone O<sub>2</sub> deprivation (Medina et al. 2009).

### 32.3.2 GENETICS

Submergence tolerance is a rare, genetically determined trait with relatively high heritability that is controlled by one or a few genes with major effects, and minor modifiers (Suprihatno and Coffman 1981, Mohanty and Khush 1985, Sinha and Saran 1988, Haque et al. 1989). Varietal differences in the degree of submergence tolerance have been noted many times (Ramiah and Rao 1953, Palada and Vergara 1972, IRRI 1978), and this genetic resource has been used in several conventional breeding programmes (Mohanty and Khush 1985). A 10 × 10 half-diallel analysis (Mohanty and Khush 1985) showed significant additive and nonadditive gene actions for submergence tolerance, but additive effects were more important. Tolerance was dominant over nontolerance and the average dominance was within the range of incomplete dominance; the Wr/Vr graphic analysis also suggested the involvement of both major and minor genes (Mohanty and Khush 1985).

In an earlier study (Suprihatno and Coffman 1985), it has been reported that at least three submergence tolerance genes are present in the four most tolerant rice varieties, namely, FR13A, Thavalu, Kurkaruppan, and Goda Heenati. The analyses of F<sub>2</sub> and backcross generations of the above four rice varieties and two susceptible (IR 42 and Nona Bokra) lines indicated the presence of a single dominant gene for submergence tolerance (Setter et al. 1997). It was also found that the first three tolerant lines possessed the same gene for submergence, but a different one in the cultivar Goda Heenati. This finding was supported by the bimodal distribution of rice lines for submergence in Thailand (IRRI 1996), but was contradicted by normal distribution noted in Philippines (IRRI 1996). The finding of the three most tolerant rice lines having the same gene for submergence



tolerance suggested that a common factor related to the tolerance of limited gas diffusion (e.g., one of the enzymes of alcoholic fermentation) may be responsible for genotypic differences in the submergence tolerance of rice (Setter et al. 1997). It also suggests that a gene for TF is involved in the expression of a multiple gene cascade that confers submergence tolerance (Setter et al. 1997).

A visual observation of symptoms is useful in attempts to identify genes or QTLs associated with tolerance, because many lines can be screened in a reasonably short time (Toojnida et al. 2003). Using this approach, QTLs for submergence tolerance have been reported on chromosomes 6, 7, 9, 11, and 12 (Xu and Mackill 1996, Nandi et al. 1997). Xu and Mackill (1996) mapped the major QTL for submergence tolerance in rice on chromosome 9. They used RAPD and RFLP markers to map submergence tolerance using a cross between a tolerant indica rice line, IR4093 I-26, and a susceptible japonica line, PI54385 I. A submergence tolerance QTL, designated as *Sub1*, was located ca. 4 cm from the RFLP marker C 1232 and accounted for 69% of the phenotypic variance for the trait (Xu and Mackill 1996).

In recent years, more than 1000 DNA markers have been mapped onto the rice genome based on RFLPs, simple sequence length polymorphisms (SSLPs), and amplified fragment length polymorphisms (AFLPs) (McCouch et al. 1988, Kurata et al. 1994, Toojnida et al. 2003). Perata and Voeselek (2006) reported that in rice, a large portion of the variation in submergence tolerance can be explained by one locus (*Sub1*, an ethylene-response-factor-like gene) on chromosome 9. This gene controls physiological and developmental processes that determine the rate of elongation when submerged (Perata and Voeselek 2006).

### 32.3.3 GENE EXPRESSION

The gene expression for anaerobic (submergence) stress is better known than other abiotic stresses (Sachs et al. 1980, Dennis et al. 1992, Setter et al. 1997). The knowledge of gene expression during anaerobiosis is due to the finding in maize that the alcohol dehydrogenase (ADH) activity increases due to flooding (Hegeman and Flesher 1960, Freeling and Birchler 1981, Freeling and Bennet 1985). The ADH activity that allows maize to survive in flooding reflects a simultaneous expression of two unlinked genes, *Adh1* and *Adh2* (Freeling 1973). The *Adh1* and *Adh2* cloned cDNA families have been identified and analyzed extensively (Dennis et al. 1984, 1985). The regions of *Adh1* and *Adh2* genes upstream from the site of transcription initiation show homology with respect to an 11 bp homologous region that includes the TATA box and three other segments of 8 bp size (Sachs and Ho 1986). The other important enzyme is pyruvate decarboxylase (PDC) (Reggiani et al. 1986), which is governed by three *Pdc* genes in rice (Reggiani et al. 1986).

During anaerobiosis, a shift in protein synthesis was reported (Sachs and Ho 1986), where it was found that there is a repression of the preexisting protein synthesis followed by a de novo synthesis of a new set of proteins. This has been reported in several crops, for instance, soybean (Lin and Key 1967), maize (Cooper and Ho 1983, Sachs and Ho 1986), rice (Bertani et al. 1981), sorghum, barley, pea, and carrot (Setter et al. 1997). The shift in protein synthesis is quite fast with a transition period of few hours. The polypeptides (33 kDa) formed during the transition period are referred to as transition polypeptides (Tps). Another group of 20 polypeptides (anaerobic polypeptides [ANPs]) is also induced after a 90 min gap, which includes isozymes of ADH (Ferl et al. 1979), glucose-6-phosphate isomerase (Kelley and Freeling 1984a), fructose-1,6-diphosphate aldolase (Kelley and Freeling 1984b), and sucrose synthetase (Setter et al. 1997). In rice, the two *Adh* genes (Xie and Wu 1989) and three *Pdc* genes have been cloned and characterized (Reggiani et al. 1986, Hossain et al. 1994, Hossain et al. 1996). Genes of these two enzymes are now being used for over- and under-expression studies in rice (Setter et al. 1997) by using the coding regions of *Adh* and *Pdc* genes through two types of promoters: constitutive (CaMV35S and Act1) (McElroy et al. 1991) and inducible (6 XARE) (Last et al. 1991).

Submergence tolerance being a complex character, it is likely that a putative single gene for submergence tolerance either (a) encodes a TF (trans-acting factor), that is, a protein or an RNA that

binds to specific regulatory DNA sequences (Singer and Berg 1991) or (b) affects the signal transduction pathway, that is, the number of steps after the submergence treatment by which the plant activates the set of genes required for survival (Dolferus et al. 1994). Around a dozen major genes known to be induced under anaerobic conditions in maize (Freeling and Bennett 1985, Bailey-Serres et al. 1988) and rice (Ricard and Pradet 1989, Rivoral et al. 1989) possess a similar sequence in their promoters (Dennis et al. 1987), and therefore suggest the involvement of a common TF (Setter et al. 1997).

Xu et al. (2006) reported a cluster of three genes at the *Sub1* locus, encoding putative ethylene response factors. Two of these genes, *Sub1B* and *Sub1C*, are invariably present in the *Sub1* region of all rice accessions analyzed. In contrast, the presence of *Sub1A* is variable. Further work identified two alleles within indica varieties carrying this gene: a tolerance-specific allele named *Sub1A-1* and an intolerance-specific allele named *Sub1A-2* (Xu et al. 2006). The phylogeny of the *Sub1* genes of the domesticated and wild rice suggests that *Sub1A* arose from the duplication of *Sub1B*, and the variation in *Sub1B* alleles is correlated with the absence or presence of *Sub1A* (Fukao et al. 2009). Fukao et al. 2009 concluded that genetic variation at the *Sub1* locus is due to gene duplication and divergence that occurred both prior to and after rice domestication.

### 32.3.4 BREEDING

The majority of loss caused by submergence is reported in the rice crop. Most *O. sativa* cultivars die within a week of complete submergence—a major constraint to rice production in south and southeast Asia that causes annual losses of over \$1 billion and affects disproportionately the poorest farmers in the world (Xu et al. 2006). Rice farming in the intensively farmed irrigated areas has become increasingly vulnerable to flooding because of the popularity of semidwarf cultivars. Therefore, breeding for submergence tolerance has been an important objective for rice breeders, and it has been felt for long that there is also a need for irrigated rice cultivars that can survive a medium depth of water (75 cm) (Toojinda et al. 2003). For these reasons, a high priority has long been set for breeding submergence-tolerant rice in the tropics utilizing the inherent variability in tolerance known to be present in the available landraces (Mackill et al. 1993).

Systemic screening at IRRI, Philippines, has resulted in the identification of flood-resistant rice cultivars, such as FR13A and FR43B from India, and Kurkaruppan and Goda Heenati from Sri Lanka (Mohanty and Khush 1985). Another submergence-tolerant elite line of IRRI, IR 49830 (-7-1-2-2), whose ancestry includes FR13A, IR42, and IR48 (Mackill et al. 1993), has been reported to have a 4–5 times higher yield than FR13A (Setter et al. 1997). Shuttle-breeding programmes between IIRI and national agricultural research institutes of several countries now provide promising advance lines as well as segregating populations for use in their target environments (Setter et al. 1997). However, the success achieved in submergence tolerance all over the world is far below our need and expectations. A probable reason for the absence of a significant introduction of improved submergence-tolerant rice cultivars during the last two decades has been the confusion between genuine submergence tolerance and shoot elongation (Maurya et al. 1988). Another reason for this fact might be the intolerance to other common stresses, like phosphorus and zinc deficiency and presubmergence drought (Neue et al. 1990). Around a decade, it was suggested (Setter et al. 1997) that although there is limited knowledge about the physiological and molecular bases of submergence stress, the association of submergence tolerance in traditional tolerant genotypes with poor agronomic traits suggests that molecular tools need to be used to enhance the submergence tolerance of rice. Since then, our knowledge has grown and the genes for submergence tolerance have been mapped in rice. There are cultivars, such as the *O. sativa* ssp. *indica* cultivar FR13A, that are highly tolerant and survive up to 2 weeks of complete submergence owing to a major QTL designated Submergence 1 (*Sub1*) near the centromere of chromosome 9 (Jackson and Ram 2003, Toojinda et al. 2003). The overexpression of *Sub1A-1* in a submergence-intolerant *O. sativa* ssp. *japonica* conferred an enhanced tolerance to the plants, the downregulation of *Sub1C*, and the upregulation of

Alcohol dehydrogenase 1 (Adh1), indicating that Sub1A-1 is a primary determinant of submergence tolerance (Xu et al. 2006). In addition, the FR13A Sub1 locus, the new variety, when introgressed into a widely grown Asian rice cultivar (Swarna) using MAS, displayed high yield and other agronomic properties of the recurrent parent and was tolerant to submergence (Xu et al. 2006).

## 32.4 HEAT TOLERANCE

Heat stress, especially during reproductive development, causes severe yield reductions in different crops. It is an important problem in tropical and subtropical environments and is believed to increase in future due to global warming (Schneider 1989, IPCC 2007, Battisti et al. 2009). Transitory or constantly high temperatures cause an array of morpho-anatomical, physiological, and biochemical changes in plants, which affect plant growth and development and may lead to a drastic reduction in the economic yield (Wahid et al. 2007). In dryland areas, heat stress may occur in association with radiation and drought stress (Loss and Siddique 1994). Plant responses to heat stress are diverse, but photosynthesis is considered the most heat-prone plant process (Bjorkman et al. 1980). Photosynthesis and respiration are more sensitive to heat stress in cool season species, such as wheat, than warm season plants (Bjorkman et al. 1980). The thermal stability of warm season species is associated with the properties of the photosynthetic system, including key enzymes and the thylakoid membrane, with the thylakoid membrane being more heat sensitive than the cell membrane (Bjorkman et al. 1980). Other reports (Bhullar and Jenner 1986, Rijven 1986) suggest that high temperature retards the conversion of sucrose to starch in developing grains (e.g., in wheat). Thus, any of a number of important metabolic functions may be disrupted due to heat stress, but a cell membrane system that remains functional during heat stress appears central to the adaptations of plants to high temperature (Raison et al. 1980). It has been suggested that the incorporation of terminal heat tolerance into high-yielding cultivars will have an energetic cost, and would require additional carbon assimilates and N inputs (Mitra and Bhatia 2008).

Understanding the molecular and physiological bases of heat tolerance in higher plants has proved difficult owing to its complexity (Blum 1988). However, other nonconventional approaches, namely, genetic engineering, are being searched to enhance the heat tolerance in plants. High-temperature tolerance has been genetically engineered in plants mainly by overexpressing the heat shock protein (HSP) genes or indirectly by altering the levels of heat shock TF proteins (Singh and Grover 2008). Apart from HSPs, thermotolerance has also been altered by elevating levels of osmolytes, increasing levels of cell detoxification enzymes, and altering membrane fluidity (Singh and Grover 2008).

### 32.4.1 GENETIC BASIS

Knowledge regarding traits conferring high-temperature tolerance and their genetics is essential for the creation of genotypes capable of giving high yields under high-temperature environments all over the world (Reynolds et al. 2007). However, no single trait can be said to be directly responsible for heat stress tolerance, though several traits are found to be associated with this mechanism. Therefore, high-temperature tolerance is characterized by measuring whole plant productivity traits (e.g., yield traits) or by utilizing bioassays in different crops (Porter et al. 1995). The traits utilized in such studies are flower bud abortion and reduction in pod fill in common bean (Shonnard and Gepts 1994); electrolyte leakage (Chen et al. 1982, Shanahan 1990), membrane thermostability (Saadalla et al. 1990), reduction of 235 triphenyl tetrazolium chloride (Towill and Mazur 1975, Porter et al. 1994), and chlorophyll fluorescence in wheat (Moffat et al. 1990); electrolyte leakage in soybean (Sullivan and Ross 1979) and common bean (Marsh et al. 1985); HSPs in sorghum (Jorgensen et al. 1993), cotton (Fender and O'Connell 1989), wheat (Vierling and Nguyen 1990), maize (Frova et al. 1980, Jorgensen et al. 1992), and pigeon pea (Devi et al. 1994); etc. Most of these traits are the measure of

the effect of the heat stress rather than the cause of heat stress tolerance. Therefore, genetics of traits causing heat stress tolerance and the heat tolerance itself are difficult to be separated. Hence, all traits are discussed below for understanding the genetics of heat stress tolerance.

A substantial genotypic variation for heat tolerance was found in groundnut, soybean, pigeon pea, and chickpea, and they were ranked from heat tolerant to heat sensitive in the same order (Srinivasan et al. 1996). Quantitative inheritance with a large environmental effect was reported for heat tolerance at pod and seed set in snap bean (Dickson and Petzoldt 1989). In another study, a single dominant gene in one snap bean accession and two genes with an epistatic effect in another were reported (Bouwkamp and Summers 1982). In a study involving two resistant and two susceptible genotypes of common bean (*Phaseolus vulgaris*) crossed in all possible combinations including reciprocals, quantitative inheritance was noted for heat tolerance (Shonnard and Gepts 1994). Additive effects were significant for two heat tolerance traits (flower bud formation and pod filling) in common bean. Cytoplasmic effects including the interaction of cytoplasmic and nuclear genes were also recorded (Shonnard and Gepts 1994). In cowpea, tolerance to inhibition of flower bud development under high temperature and for a long day was due to a recessive gene (Hall 1992), while tolerance during pollen formation was under the control of a single dominant gene (Marfo and Hall 1992). In tomato (*Lycopersicon esculentum*), it was reported that heat tolerance during fruit set was conferred by a few partially dominant genes, but narrow sense heritability was very low (8%) due to large environmental effects (Shelby et al. 1978).

Genetic effects on membrane thermostability in wheat in 90 F<sub>2</sub> derived lines of heat-tolerant and susceptible lines showed that heat tolerance is not simply inherited (Saadalla et al. 1990). Genetic differences in membrane thermostability were noted in soybean (*Glycine max* L. Merr.) (Wallner et al. 1982).

Marsh et al. (1985) examined inheritance of membrane stability in common bean and found that heat tolerance was under the control of few genes. They also found low additive effects along with epistasis. In a diallel including a reciprocal of six wheat genotypes, significant general combining ability effects and maternal effects were noted (Moffat et al. 1990). On the basis of the relation of heat stress effect and the reduction of 235 triphenyl tetrazolium chloride, which produces a red formazan, significant differences in acquired high-temperature tolerance were reported in wheat (Porter et al. 1994). Using 20 F<sub>1</sub> progenies, produced through a complete 5 × 5 diallel mating design of tolerant and susceptible genotypes, showed that only the general combining ability effect was highly significant accounting for 67% of the total genetic variation (Porter et al. 1995).

There is extensive evidence of both qualitative and quantitative intraspecific genetic variability for low-molecular-weight (LMW) HSPs in crops, for example, sorghum (Jorgensen et al. 1993), cotton (Fender and Connell 1989), wheat (Towill and Mazur 1975, Vierling and Nguyen 1990), and maize (Jorgensen et al. 1992). Very few reports are available regarding HSP gene transmission in plants. Additive inheritance for some HSPs has been reported in barley, where the presence of hybrid-specific HSPs indicated the activation of genes that were suppressed in one parent (Marmioli et al. 1989). Both additive and nonadditive inheritance was demonstrated in the F<sub>1</sub> hybrids of maize (Frova et al. 1988). Intraspecific qualitative polymorphism in LMW synthesis is extremely rare (Ottaviano et al. 1991), and quantitative variation in HSP synthesis may determine relative thermal tolerance levels (Frova and Gorla 1993). A genetic analysis of HSPs in maize, HSP synthesis, revealed both qualitative and quantitative polymorphism implicative of differences in HSP structural genes and regulatory factors (Jorgensen and Rosenow 1995). The F<sub>1</sub> hybrid HSP profile indicated that the synthesis of all parental HSPs conformed to dominant inheritance patterns, including complete dominance, overdominance, and codominance; there was evidence for unlinked loci of four different HSP gene pairs, but data for three other HSP gene pairs were inconclusive (Jorgensen and Rosenow 1995).

### 32.4.2 GENE EXPRESSION

Heat stress is known to induce a set of proteins called heat shock proteins (HSPs) (Key et al. 1981). HSPs are known to be associated with acquired thermal tolerance in many species, including bacteria (Sanchez and Lindquist 1990); mammalian fibroblasts (Riabowol et al. 1988); and higher plants

such as soybean (Lin et al. 1984, Kimpel and Key 1985a), wheat (Krishnan et al. 1989), cotton (Burke et al. 1985), and maize (Jorgensen et al. 1992). In plants, a heat shock of 8°C–10°C above the normal growing temperature induces the synthesis of both high (65–110 kDa) and low (15–27 kDa) molecular HSPs, with the LMW proteins being the most prevalent (Kimpel and Key 1985b, Mansfield and Key 1987). A subset of the LMW group, 15–18 kDa, is unique to higher plants (Sachs and Ho 1986).

The LMW HSPs are a complex group, with as many as 30 members present in soybean (Nagao et al. 1985). The induction of LMW HSPs has been well documented (McElwain and Spiker 1992) and their number is known in some monocot species (Mansfield and Key 1987). The detection of low- and high-molecular-weight HSPs synthesized in seedlings and flag leaves in flowering plants suggest that HSPs are synthesized before leaf temperatures reach levels that are considered injurious to growth and development (Hendershot et al. 1992).

HSPs are among the fastest known gene expressions in plants (Ougham 1987). Heat shock response may be the accumulation of damaged proteins (Munro and Pelham 1985). This is supported by the fact that the small protein ubiquitin, which has a role in the ATP-dependent breakdown of abnormal proteins (Hershko and Ciechanover 1982), is itself an HSP (Bond and Schlesinger 1985).

Limited information is available about structural relationships among the HSP genes and the molecular regulation of their transcription and translation to protein (Frova et al. 1988, Leonardi et al. 1988, Jorgensen and Rosenow 1995). On the basis of nucleotide and amino acid similarities and protein localization, there are four families of structural LMW HSP genes known in plants (Vierling 1991); one each encoding plastid localization and endomembrane proteins, and two that encode cytoplasmic proteins (classes I and II). The HSPs of 17–18 kDa comprise classes I and II. There are several class II gene sequences (Dietrich et al. 1991, Goping et al. 1991) as well as class I cDNA HSPs (Jorgensen and Nguyen 1994).

### 32.4.3 BREEDING

Increasing productivity under heat stress conditions requires the development of heat-tolerant genotypes in all crops. The improvement of heat stress tolerance can contribute to sustainability and provides a way to extend the area under cultivation (Srinivasan et al. 1996). Limited progress has been made with regard to breeding heat-tolerant genotypes, probably because yield losses due to heat are more subtle than biotic stresses (Summerfield et al. 1984). The two most important hurdles are the absence of substantial information on the genetic diversity for heat tolerance traits and effective screening techniques (Wery et al. 1994). Both *in vivo* and *in vitro* methods are used for screening, but *in vitro* methods are advantageous in that they are plant conserving, and this feature is important in plant breeding for heat tolerance (Srinivasan et al. 1996).

The genetic control of heat tolerance is poorly understood (Ortiz et al. 2008a,b). However, the level of tolerance to high temperature varies among genotypes (Blum 1986, Shipler and Blum 1986, Moffat et al. 1990, Pfeiffer et al. 2005), suggesting that the trait can be improved (Moffat et al. 1990). The indication of a larger additive genetic variation with regard to some traits believed to be associated with heat stress also indicates that gains from selection for improved heat tolerance should be possible. Resistance to high temperature involves complex tolerance and avoidance mechanisms. However, the cell membrane is believed to be a site of primary physiological injury by heat (Blum 1988). The damage to a membrane can be measured by estimating solute leakage from the cell. Since membrane thermostability is reasonably heritable (Fokar et al. 1998) and shows a high genetic correlation with the yield, it has potential application in breeding, but does require a laboratory methodology to make measurements (Reynolds et al. 2001a,b).

Breeding for heat tolerance in cowpea has involved a pedigree breeding approach with selection beginning in the  $F_2$  generation (Hall 1990) to incorporate major recessive genes conferring heat tolerance during early floral bud and seed coat development (Patel and Hall 1988). It has been

suggested that to overcome difficulties caused by environmentally induced variation, incorporating heat tolerance pod set into other genetic backgrounds will require family selection in advanced generations to ensure that trait is fixed (Marfo and Hall 1994). Recurrent selection has been suggested to accumulate genes favoring high-temperature tolerance based on chlorophyll fluorescence measurements in wheat (Moffat et al. 1990).

In many countries and environments, late planting can expose the crop and breeding nurseries to high temperatures from flowering onward, giving wheat breeders the opportunity to select lines with high levels of heat tolerance (Ortiz et al. 2008a,b). At CIMMYT, lines are selected during the segregating phase for adaptation to heat by planting late, and a gravity table is used to separate bulk populations into those that can maintain seed weight under high temperature; the derived lines are then tested under heat stress in yield trials (Ortiz et al. 2008a,b). Physiological tools, such as the infrared thermometer that measures canopy temperature depression (CTD), are also available to assist the plant breeder in discriminating among progenies (Reynolds et al. 1998). Heat avoidance or early maturity is an extremely important trait to circumvent the effects of high temperature at grain filling (Ortiz et al. 2008a,b). All currently grown popular cultivars in the eastern Gangetic Plains are earlier-maturing than cultivars popular in the northwestern Gangetic Plains. A simultaneous improvement of heat tolerance and yield potential of earlier-maturing germplasm is the best option to increase production in heat stress environments, and is being practiced (Joshi et al. 2007a–e). One of the most popular wheat varieties of India, HUW234, which currently occupies more than three million hectares of area, possesses unique features of both avoidance and tolerance of heat stress of the northeastern plains zone of the country. Its early maturity, profuse tillering, high grain number per spike, and fast ripening provides it a clear superiority over other varieties under late- to very-late-sown conditions when hot winds of early summer cause serious yield losses.

## 32.5 COLD TOLERANCE

Low temperature, especially in the northern region of the temperate climatic zone, presents substantial obstacles to the survival of plants throughout the winter (Andrews 1997) and is one of the most severe stresses that limits crop growth and productivity (Boyer 1982). Reduction in grain yield is incurred not only as a direct result of winter damage but also as a result of limiting the areas where such crops can be sown (Steponkus 1978). Although low-temperature (LT) stresses are usually of two types, chilling above 0° temperature and freezing at subzero temperature (Welin et al. 1996), the winter may expose young seedlings to many kinds of stresses (as in wheat), such as direct frost effect, cold winds, snow cover, intense freezing and glaciation of the soil, and frost lifting in spring. In grasses and wheat, there are two different mechanisms of tolerance to ice encasement, that is, rapid (wheat) and slower (grasses) glycolysis (Andrews 1997). Following cold acclimation, a number of forage species are highly tolerant to extreme cold conditions of ice encasement (Gudleifsson et al. 1986), even greater than winter wheat (Andrews 1997). In grasses, berrings hairgrass has recently been shown to have an extremely high tolerance to anoxia (Crawford and Braendle 1996), a property that is common to many arctic plants (Andrews 1997). The physiological and biological processes that lead to cold tolerance or the adaptation of plants to low temperature are extremely complex (Paldi et al. 1996a).

### 32.5.1 MORPHOPHYSIOLOGICAL TRAITS: GENETIC BASIS

Like other abiotic stresses, tolerance to low temperature is also due to the joint action of several traits of plants. Significant correlations were established between cold hardiness and day to heading (Fowler and Carles 1979), and the growth habit (Hayes and Aamodt 1927, Quisenberry 1931, Brule-Babel and Fowler 1988) in wheat. In general, spring wheat lines are less hardy than winter lines and spring growth habit is dominant over winter growth habit. Winter growth habit of wheat is possibly inherited by a *Vrn* (vernalization requirement) gene (Brule-Babel and Fowler 1988). Chahalan

and Law (1979) found no evidence of genetic linkage between cold hardiness and vernalization requirement in wheat even though chromosomes in the homologous group 5 were implicated in the control of both of these characters. Studies in grass species (e.g., clover) also do not show correlation between a single trait and cold tolerance (Smith 1949, Ronningen 1953, Annicchiarico and Piano 1995). Photoperiodism, an important trait for adaptability in cold climate, is governed by genes present in the group 2 chromosome of wheat (Welsh et al. 1973). At least three genes *Ppd1*, *Ppd2*, and *Ppd3* governing photoperiodism are known in chromosomes 2D, 2B, and 2A, respectively (Worland et al. 1987). Low temperature has been found to enhance the anthocyanin synthesis in plants such as sorghum, cabbage, maize, Arabidopsis, apple, roses, and petunia (Shvarts et al. 1997).

### 32.5.2 GENETICS

Winter hardiness is a genetically programmed integrated process (Weiser 1970, Sutka and Veisz 1988). The two major components of freezing stress resistance are freezing tolerance in the nonacclimated state (normal growing condition) and the capacity to cold-acclimate (increase in freezing tolerance) upon exposure to chilling temperatures (Palta and Simon 1993). Genetics of winter hardiness was attempted as early as 1912 when Nilsson Ehle (Nilsson-Ehle 1912) of Sweden investigated winter hardiness in wheat, and on the basis of the appearance of transgressive segregants in a cross of two cultivars intermediate in winter hardiness, he reported that it is a quantitative trait under the control of a polygenic system (Nilsson-Ehle 1912). Since then, no general opinion has arrived on this issue. It has been reported to be recessive (Rosenquist 1933), intermediate (Lyfenko 1979, Erickson 1980), dominant (Rosenquist 1933), or overdominant (Rosenquist 1933, Kir'yan and Barashkova 1981). It has been reported that winter hardiness is under the control of dominant genes in mild cold, while under severe cold it is governed by recessive genes (Quisenberry 1931, Gullord et al. 1975, Sutka 1981). Winter hardiness behaved as a recessive factor as early as 1923 in a cross made by Schafer (1923).

A majority of studies indicate a polygenic control of cold tolerance (Hayes and Aamodt 1927, Worzella 1935, Parodi et al. 1983, Limin and Fowler 1988, Norell et al. 1986). The quantitative nature of winter hardiness is also supported by the absence of a drastic improvement in the winter hardiness of different crops (Quamme et al. 1972), the appearance of transgressive segregants (Smith 1949), and a complex of factors influencing winter hardiness (Levitt 1972). However, all the genes do not work together and different genes affect tolerance at different levels of stress (Gullord et al. 1975). This is evidenced by studies showing that winter hardiness genes may act as dominant or recessive depending on the type of environment (Quisenberry 1931, Worzella 1935, Muehlbauer et al. 1970, Sutka and Veisz 1988). In wheat, 11 chromosomes carry genes for cold tolerance with chromosome number 5 being the most important (Thomashow 1990). Some studies have implicated 15 of 21 chromosome pairs of wheat to be associated with cold tolerance (Sutka 1981) with chromosome 5A (Cahalan and Law 1979, Sutka and Kovacs 1985), 7A, 4D, and 5D (Law and Jenkins 1970, Sutka 1981) most commonly mentioned. In barley, a major QTL was found associated with the 7th chromosome (Hayes et al. 1993, Pan et al. 1994). In *Solanum* species, the nonacclimated freezing tolerance and acclimation capacity were found to be separate heritable traits controlled by few genes (Stone et al. 1993). In rye (*Secale cereale*), cold hardiness is controlled by genes mainly with additive effects (Shanahan et al. 1990).

The genetics of frost tolerance studied in winter wheat by using a complete diallel (Gullord et al. 1975, Briggie, Sutka 1994) showed that frost tolerance is controlled by an additive dominance system (Puchkov and Zhirov 1978, Sutka 1981, 1984). Several studies (Sutka 1984, Sutka and Veisz 1988, Sutka 1989, Sutka 1994) have shown that frost tolerance is a complex character controlled by at least 10 of the 21 pairs of chromosomes (Sutka and Veisz 1988); chromosomes 5A and 5D have been implicated most frequently and they appear to carry major genes (Sutka 1994). The gene for frost resistance (*Fr1*) was located on the long arm of chromosome 5A (Roberts 1990, Sutka 1994), and there might be close genetic linkage between *Vrn1* and *Fr1*

(Roberts 1990). Studies done so far indicate the presence of four major genes for vernalization requirement: *Vrn1*, *Vrn2*, *Vrn3*, (Pugsley 1972, 1973), and *Vrn4* (Pugsley 1973). Another gene *Vrn5* was also reported (Law 1966).

Cold tolerance is under the control of both additive and nonadditive gene effects in chickpea (Malhotra and Singh 1990) and pea (Markarian and Anderson 1966, Auld et al. 1983). Genetic interactions also play an important role in cold tolerance (Malhotra and Singh 1990). Three additive genes or linkage groups are reported to control winter hardiness in pea (Liesenfeld et al. 1986). The expression of LT tolerance has been found to be under the control of same genetic factors in the sporophyte and gametophyte of potato (Zamir et al. 1981, 1982, Zamir and Vallejos 1983). High-heritability estimates for cold hardiness have been reported in wheat (Sutka 1981, Sutka 1984), barley (Rohde and Pulham 1960, Eunus et al. 1962), and oats (Muehlbauer et al. 1970). One of the important conclusions from a large number of studies was that cold acclimation includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury (Thomashow 1999). In addition, a family of *Arabidopsis* TFs, the CBF/DREB1 proteins, was identified that controls the expression of a cold-induced gene that increases plant freezing tolerance (Thomashow 1999).

In potato, Stone et al. (1993) demonstrated that freezing tolerance and the ability to cold-acclimate are under independent genetic control. This was later confirmed in other plant species (Teutonico et al. 1995, Arora et al. 1998). These results have important implications for the improvement of cold tolerance of cultivated potatoes. For a successful improvement of frost hardiness, both components must be transferred to the cultivated potatoes (Palta and Simon 1993).

### 32.5.3 GENE EXPRESSION

The molecular mechanism that regulates cold tolerance is not sufficiently well known (Crawford and Braendle 1996). Weiser, in 1970 (Weiser 1970), suggested that cold acclimation might involve changes in the gene expression. Since then, however, more and more information has been reported in this field (Guy et al. 1985, Houlde et al. 1972, Paldi et al. 1996b). The realization that cold acclimation requires an altered expression of tolerance-related genes not seen under nonacclimating conditions is the basis for the isolation and characterization of cold-induced genes (Paldi et al. 1996b). Several studies have demonstrated that plants synthesize a new set of proteins when exposed to a cooler environment (Guy 1990). The existence of partially different mRNA populations in nonacclimated and acclimated plants has allowed the isolation of cDNAs corresponding to acclimation-specific mRNAs by differential screening as in alfalfa (Mohapatra et al. 1987, Mohapatra et al. 1989), *Arabidopsis* (Kurkela and Frank 1990), and barley (Cattivelli and Bartels 1990, Berkel et al. 1994, Welin et al. 1996). The temporal pattern of LT-induced gene activation varies between different plant species ranging from few hours (*A. thaliana*) (Hajela et al. 1990, Nordin et al. 1991, Yamaguchi-Shinozaki and Shinozaki 1993, Welin et al. 1994) to several days (Mohapatra et al. 1987, Monroy et al. 1993, Wolfrain et al. 1993).

LT-responsive genes are transcriptionally regulated through sequence-specific TFs that bind to their target sequence on the corresponding promoters (Welin et al. 1996). LT-induced genes contain certain sequence elements that resemble ABA-responsive (Palva et al. 1994) and drought-responsive elements (120). Homologous regions are also present in the promoters of LT-induced genes: *cor15a* (Baker et al. 1994), *rab18*, *kin1*, *kin2* (Palva et al. 1994), *lti29*, and *cor47* (Welin et al. 1996).

The identification of an mRNA group that only functions during a cold effect and codes proteins found only in frost-resistant wheat varieties (Perras and Sarhan 1989) suggests a positive correlation between the quantity of proteins synthesized during the cold effect and the frost resistance of the varieties (Paldi et al. 1996b). Some mRNAs decline during exposure to low temperature, as in *Brassica* (Anderson et al. 1994), rice (Hahn and Walbot 1989), and spinach (Guy 1990).



The cold-induced rRNA synthesis in wheat takes place in seedlings as a result of low temperature during the first few days of cold treatment (Paldy and Devay 1977, Devay and Paldy 1990), and the cold-induced rRNA synthesis is closely correlated to the rRNA cistron number (Devay and Paldy 1977). Quantitative and qualitative changes have been noted in the rRNA maturation processes due to low temperature in the weak frost resistant line of wheat, as a consequence of which there is an increase in the last precursors (1.4 and 0.9MD) of the two stable cytoplasmic rRNAs (Paldi et al. 1996b).

Freezing tolerance includes tolerance to freeze-induced dehydration (Guy 1990, Levitt 1980). This is further substantiated by the fact that several of the LT-induced proteins are similar to proteins induced in response to water stress (dehydrins) (Baker et al. 1988, Mundy and Chua 1988). Certain proteins having the putative protein-stabilizing function (*Bip*, *Hsp70*, and *Hsp90*) have been identified among LT-induced proteins (Anderson et al. 1994, Kishor et al. 1995). Cryoprotective polypeptides capable of protecting the plant thylakoid membrane in vitro against a freeze-thaw cycle have also been reported in cabbage and spinach (Hincha et al. 1989, 1990). The structural similarity of the gene product of *kin1* from *A. thaliana* with an antifreeze protein of swinter flounder (Kurkela and Frank 1990) led to the speculation of the presence of the antifreeze protein, to be contradicted by Thomashow (1993). However, there are reports of the presence of an antifreeze protein in winter rye (Griffith et al. 1992, Urrutia et al. 1992).

In *A. thaliana* leaves, pigment accumulation in response to low temperature results from the activation of phenylalanine ammonia-lyase (*pal*) and chalcone synthase (*chs*) gene transcription in a light-dependent manner (Leyva et al. 1995). It was suggested that light dependency is a general feature of the cold-induced gene expression. However, in *Petunis corolas*, cold activation of the *chs* expression was not light dependent (Shvarts et al. 1997). The effect on the *chs* expression was not always correlated with that on the anthocyanin content, suggesting a posttranslational effect (Shvarts et al. 1997). Earlier, Christie et al. (1994) suggested that the effect of temperature is associated with transcription, transcript stability, translation, and enzyme activity. Low temperatures do not simply create conditions that facilitate the developmental activation of the *chs* expression; they act as a separate inducing signal (Christie et al. 1994, Shvarts et al. 1997). The transduction of an LT signal (2°C–5°C) for the activation of a cold-acclimation-specific (*cas*) gene has also been studied, which probably does not belong to the *chs* group (Shvarts et al. 1997).

The transcripts of enzymes of the fermentation pathway, ADH and PDC, have been found to increase as a result of the hypoxic acclimation in wheat (Waters et al. 1991) and maize (Andrews et al. 1994). However, more tolerant forage grasses, timothy (*Phleum pratense*) and berrings hairgrass (*Deschampsia berengensis*), show lesser activity of ADH and PDC (Andrews 1997).

Desiccation often accompanies cold acclimation and freezing stress (Shvarts et al. 1997); therefore, at the molecular level, genes induced by water stress and ABA are also induced by cold stress in barley, rice, and spinach (Hahn and Walbot 1989). In contrast, genes induced by cold temperature can respond to water stress and ABA (Sutka 1981, Frank 1990, Berkel et al. 1994). The homology between HSPs and cold-induced proteins has been reported in potato (Berkel et al. 1994).

#### 32.5.4 BREEDING

Conventional breeding has been utilized by breeders all over the world to enhance cold tolerance in different crops. Handling a complex trait, such as winter hardiness, in a breeding program is a difficult task, due to the large number of genes involved and the numerous interactions with the environment (Săulescu and Braun 2001). But the main difficulty in breeding a cold-tolerant line in crops such as wheat is that high freezing tolerance is generally associated with lower yields and, later, maturity (Săulescu and Braun 2001). Since many traits are associated with the freezing tolerance and every additional breeding objective is expected to slow down the genetic progress for all other traits of interest, the breeding objective should not be to maximize winter hardiness, but to develop cultivars with the minimum winter hardiness necessary for a given target area (Săulescu

and Braun 2001). This is supported by the fact that, in general, the most successful winter wheat cultivars have only marginally greater winter hardiness than the minimum required for the area in which they are grown (Fowler et al. 1981).

Sutka (1994) suggested three ways to improve genetic variation for frost tolerance in wheat: interspecific crossing, chromosome manipulation, and the induction of somaclonal variation. Wild species related to cultivated wheat, for instance, *Ae. cylindrica*, *Agropyron glaucum* (*intermedium*), and *Agropyron elongatum*, are an extremely promising source of increased genetic variation (Sutka 1994) for cold tolerance. Disomic additions of *A. glaucum* were able to survive freezing to a temperature as low as  $-18^{\circ}\text{C}$ . A somaclone of wheat was significantly better than control for cold tolerance, and thus, of practical importance (Hinch et al. 1989). It has been suggested that in chickpea, selection would be more effective if dominance and epistatic effects were reduced after a few generations of selfing (Malhotra and Singh 1990).

The in vitro selection through the anther culture has been suggested as a useful tool for breeding LT tolerance in crops (Zamir et al. 1981, 1982, Zamir and Vallejos 1983) on the ground in that there is genetic overlap between the sporophyte and the gametophyte (Mulcahy 1979). Gametophytic selection for LT tolerance has been successfully demonstrated in tomato (Zamir and Gadish 1987), maize (Kovacs and Barnabas 1992, Krisjansdottir 1993, Lyakh and Soroka 1993), and potato (Lynch and Steponkus 1987).

Despite a great progress in understanding the molecular basis for plant cold acclimation, the complexity of the system hampers the genetic engineering of plants having freezing tolerance (Welin et al. 1996). Among the possible approaches to enhance cold tolerance in plants, the ways that hold promise are (Welin et al. 1996) (1) increasing the freezing resistance of plant plasma membrane by increasing the amount of phospholipid (Lynch and Steponkus 1987, Palta and Weiss 1993), as showed successfully in tobacco (Murata et al. 1992, Kodama et al. 1994); (2) metabolic alterations alleviating the detrimental effects of desiccation stress, for example, OA through osmoprotectants (Welin et al. 1996); (3) exploiting cryoprotective and antifreeze proteins, for example, fish antifreeze proteins (Hightower et al. 1991); and (4) manipulation of signal pathways leading to the expression of tolerance genes.

For several years, it has been felt that limited success has been achieved using traditional plant-breeding methods to improve the freezing stress resistance in crops (Marshall 1982, Palta and Simon 1993, Vega et al. 2003). Even crops that have undergone extensive breeding, such as winter wheat, have not had significant improvements, and cultivars that were released more than 50 years ago remain among the most cold-hardy today (Limin and Fowler 1991). Recent progress in plant breeding has focused on the use of molecular marker techniques to facilitate cloning and efficient introgression of favorable genes through marker-assisted selection (Lande and Thompson 1990, Dudley 1993). Genomic regions with a significant effect on the freezing tolerance have been detected in several crops: *Citrus* (Cai et al. 1994), *Brassica* (Teutonico et al. 1995, Kole et al. 2002), *Triticum* (wheat) (Sutka 1994), *Eucaliptus nitens* (Byrne et al. 1997), *Lycopersicon* (tomato) (Foolad et al. 1998), *Vaccinium* (blueberry) (Rowland et al. 1999), and *O. sativa* (rice) (Saito et al. 2001).

## 32.6 SALINITY RESISTANCE

Soil salinity is a major agricultural problem, particularly in irrigated agriculture. Around 10% of the world's arable land is affected by salinity (Tanji 1990, Shanon 1997), while it is a serious problem in around 20% of irrigated agricultural land (Flowers and Yeo 1995). Another report (<http://www.plantstress.com/Articles/index.asp>- October 22, 2007) suggests that more than 6% of the world's land (19.5% of irrigated land plus 2.1% of dryland agriculture) is now affected by salinity of various magnitudes (Kader and Lindberg 2008). One-third of the land in Australia is salt affected (Northcote and Skene 1972), while in India and Pakistan, such areas constitute around 5% of their total cultivable land. In irrigated areas, the percentage of salt-affected land is much higher. In the

United States, 23% of irrigated land is under salt stress (Shanon 1997). With as much as half of the world's existing irrigation systems under the influence of secondary salinization, alkalization, and water logging (Szabolcs 1987), the coexistence of irrigation and salinization threatens current agricultural productivity (Flowers and Yeo 1995). Despite such importance of salt-affected areas in world agriculture, too little progress has been made in improving the salt tolerance of crops (Flowers and Yeo 1995, Shanon 1997). The problem is expected to increase in the coming future, and an integrated approach seems to be the only answer. Genetic engineering is also being seen as a potent tool to handle this problem through the overexpression of  $\text{Na}^+/\text{H}^+$  antiporters in plants (Zhang et al. 2004).

### 32.6.1 MORPHOPHYSIOLOGICAL TRAITS: GENETIC BASIS

Traits associated with salt tolerance have been investigated by several workers (Yeo and Flowers 1989, Noble and Shannon 1990, Tanji 1990, Yeo et al. 1990, Foodlad 1997, Shanon 1997), but exact traits are still to be identified (Tanji 1990). The differential response of plants to salt stress at different growth stages has added further problems in this direction. In wheat and sorghum, salt tolerance is associated with seed size, with larger seed having greater tolerance (Amthor 1983, Grieve and Francois 1992). Seedling survival in saline solution is also an indicator of salt tolerance of crops, and has been studied in *Medicago* (Allen et al. 1985, Al-Khatib et al. 1993), forage crops (Ashraf et al. 1987), and potato (Jefferies 1994). The results showed that characters underlying short-term tolerance may contribute to long-term tolerance, but did not themselves confer long-term tolerance (Jefferies 1996).

Two broad physiological mechanisms by which plants respond to salt stress (Foodlad 1997) are (1) the inclusion and use of inorganic ions as osmotica to maintain a favorable water balance (halophytic response), and (2) a partial exclusion of ions and the synthesis of organic solutes for OA (glycolytic response) (Greenway and Munns 1980, Curatero 1992). Salt tolerance is associated with an increased capacity of ion regulation through compartmentation and transport of toxic ions, OA, and maintenance of membrane integrity (Yeo 1983). Most of the crops respond to salt stress by excluding ions from the shoot, and genetic variation exists for the threshold level at which the exclusion mechanism fails (Foodlad 1997). Physiological and genetic factors that contribute to the growth of glycophytes at a very high salt concentration are related to the survival more than the yield potential, and hence, are of little interest to growers except those engaged in subsistence agriculture (Shannon and Noble 1990). Low sodium transport has been suggested as an important heritable trait for salt tolerance in rice (Yeo 1992, Garcia et al. 1995).  $\text{Cl}^-$  exclusion was responsible for the genetic difference for salt tolerance in soybean (Abel and Mackenzie 1963), and was found a heritable character in white clover (*Trifolium repens*) and lucern (*Medicago sativa*) (Noble and Shannon 1990). Under salt stress, the tolerant tomato genotype accumulated significantly less  $\text{Na}^+$  and  $\text{Cl}^-$  and more  $\text{Ca}^{2+}$  than the leaves of the sensitive genotype (Foodlad 1997). A generation mean analysis indicated that under salt stress, both the absolute and relative growth and the  $\text{Na}^+$  and  $\text{Ca}^{2+}$  accumulation in the leaf were genetically controlled with additivity being the major genetic component (Foodlad 1997); a moderate estimate of narrow sense heritability ( $0.49 \pm 0.09$ ) was obtained for the shoot dry weight under salt stress treatment (Foodlad 1996).  $\text{K}^+$  selectivity was identified to be a principal adaptive mechanism to salt stress in legumes and cereals (Lauchli 1984, Dvorak and Gorham 1992).

Under saline conditions, the ability to keep a low cytosolic  $\text{Na}^+$ -concentration appears to be an important trait of salt-tolerant plants (Flowers and Hajibagheri 2001, Maathuis and Sanders 2001, Carden et al. 2003, Golldack et al. 2003, Kader and Lindberg 2005, Anil et al. 2007). Plant cells can maintain a low cytosolic  $\text{Na}^+$ -concentration, either by restricting  $\text{Na}^+$  influx into the cell, or by extruding cytosolic  $\text{Na}^+$  into the apoplast or vacuole, or by both (Kader and Lindberg 2008). Another report (Anil et al. 2007) suggests lesser permeability of the plasma membrane to  $\text{Na}^+$  in the salt-tolerant rice cv. Pokkali compared to that in the salt-sensitive rice cv. Jaya.

### 32.6.2 GENETICS

The degree to which different plants can tolerate high concentrations of salt in their rooting medium is under genetic control (Epstein and Jefferies 1964, Allen et al. 1985, McNeilly 1990, Shanon 1990). Genetics of salt tolerance has been investigated in several crops, and the results so far indicate monogenic to polygenic control. Salt tolerance was recorded as a heritable trait in *Agropyron intermedium* (Hunt 1965) and barley (Norlyn 1980). In sorghum, genetic variation for OA was studied in 10 inbred lines and variation was noted due to more than a single gene, and both general and specific combining ability effects were found significant (Basnayake et al. 1994). The greater tolerance of wild sunflower *Helianthus paradoxus* is due to a single dominant gene *Sa<sub>1</sub>*, but a modifier may also be present (Miller 1995). Salt tolerance in wheat grass showed that tolerance behaves in an additive fashion (Weimberg and Shanon 1988). In tomato, a stage-specific polygenic control was suggested to control salt tolerance (Shanon 1982, Jones and Qualset 1984, Jones 1987). In another study, a generation mean analysis showed that the additive gene action was the predominant component for salt tolerance in tomato; the narrow sense heritability was estimated to be moderately high (Foolad and Jones 1991). Six marker loci in tomato have shown association with QTLs involved in yield under salinity (Breto et al. 1994).

The *Gpert* ("Golden Promise" erectoides) mutation, produced by gamma-ray irradiation in the barley variety Maythorpe in the late 1950s, which is allelic to *ari-e* mutants (short awned, *breviaristatum*), has a significant effect on salt tolerance (Froster et al. 1994). *Gpert* performs similar to other *ari-e* mutants (*ari-e.1*, *ari-e.119*, *ari-e.156*, and *ari-e.228*) and possesses a relatively low shoot Na<sup>+</sup> content and a higher salt tolerance index (Pakniyat et al. 1997a) in comparison to nonmutants. These mutants show greater tolerance than *denso* (*sdw*) or *ert-k<sup>32</sup>* dwarfing mutants (Froster et al. 1994, Pakniyat et al. 1997a). The *Gpert* mutation is modified by a genetic background (Pakniyat et al. 1997b) and is also associated with drought tolerance characters.

In saline environments, bread wheat, *T. aestivum* (genomes AABBDD), accumulates less Na<sup>+</sup> and more K<sup>+</sup> on expanding and young leaves than durum wheat, *T. turgidum* (genomes AABB) (Dubcovsky et al. 1996). Chromosome 4D accounts for 50%–60% of the difference between bread wheat and durum wheat for this trait (Gorham et al. 1987, Dvorak and Gorham 1992). Dvorak and Gorham (1992) recombined chromosome 4D with durum wheat chromosome 4B by using the *ph1c* mutant of durum wheat and found that K<sup>+</sup>/Na<sup>+</sup> discrimination is controlled by a single locus on the long arm of chromosome 4D, which was designated *Knal*. The *Knal* locus was mapped on a short region in the 4DL arm and was completely linked to *Xwg199*, *Xabc305*, *Xbcd402*, *Xpsr567*, and *Xpsr375* (Dubcovsky et al. 1996). The 5J chromosome of *Agropyron junceum* carries a major dominant gene(s) conferring tolerance to salt (Forster et al. 1988). Stølen and Andersen (1978) found that tolerance to high soil acidity is controlled by a single dominant gene, designated *Pht*, on chromosome 4. Subsequently, genes with positive effects for salt tolerance were located to chromosomes 4H and 5H of *Hordeum vulgare* and 1H<sup>ch</sup>, 4H<sup>ch</sup>, and 5H<sup>ch</sup> of *Hordeum chilense* (Forster et al. 1990). In a study, Minella and Sorrells (1997) reported that the aluminum tolerance gene (*Alp*) is distally located from the centromere on chromosome 4, suggesting that tolerance to low pH (*Pht*) and aluminum tolerance are controlled by the same locus.

Salt tolerance at the cellular level involves several hundreds of stress-responsive genes for ionic homeostasis as well as osmotic homeostasis (Bartels and Sunkar 2005, Chen and Zhu 2005, Sreenivasulu et al. 2007). Recently, Huang et al. (2009) cloned and characterized DST (drought and salt tolerance) in rice crop. This was a previously unknown zinc finger TF that negatively regulates stomatal closure by a direct modulation of genes related to H<sub>2</sub>O<sub>2</sub> homeostasis, and identifies a novel pathway for the signal transduction of DST-mediated H<sub>2</sub>O<sub>2</sub>-induced stomatal closure (Huang et al. 2009). Loss of the DST function increases stomatal closure and reduces stomatal density, consequently resulting in enhanced DST in rice (Huang et al. 2009). This provides a new opening to understand the mechanism of stomata-regulated abiotic stress tolerance, and therefore, could lead to an important genetic engineering approach for improving salt tolerance.

### 32.6.3 GENE EXPRESSION

The adaptation of plants to a saline environment must be due to some salt-related changes in the pattern of gene(s) expression (Foodlad 1997). More than 100 genes were estimated to be expressed when subjected to salt stress (Meyer et al. 1990). There are several reports of alterations in protein accumulation due to salinity (Meyer et al. 1990, Yeo et al. 1990, Jain et al. 1993). One of the most characterized genes associated with salt tolerance is the gene encoding a 26 kDa protein, called osmotin, which is responsive to several environmental and hormonal signals, including osmotic and pathogen stress (Singh et al. 1985, Singh et al. 1986, Singh et al. 1987, King et al. 1988, LaRosa et al. 1989). Osmotin gene expression and protein accumulation were elicited vegetative tissues of tomato in response to short- or long-term exposure to NaCl as well after severe water loss (Grillo et al. 1995). This gene is also stimulated by ABA (Singh et al. 1987, Skriver and Mundy 1990). Although NaCl can induce the osmotin gene through changes in ABA levels, this signal can also regulate osmotin mRNA accumulation by ABA-independent signal transduction pathways, as suggested for the *Em* gene in rice (Bostock and Quatrano 1992), and other ABA-inducible mRNAs from wheat (Morris et al. 1990) and rapeseed (Finkelstein and Crouch 1986). A cis-deletion analysis of the osmotin promoter indicated that the induction by NaCl, ABA, and ethylene is associated with the same region of the promoter (Raghothama et al. 1993). Many molecular responses to salt stress in a common ice plant (*Mesembryanthemum crystallinum*) are elicited primarily by the transcriptional induction of specific genes (Cushman et al. 1989, Andolfatto et al. 1994).

Several classes of transporters are reported to be required in regulating sodium homeostasis under salt stress (Zhang et al. 2004). Arabidopsis has played a vital role in many investigations of the basic processes that occur during salt stress (Kader and Lindberg 2008). High-affinity potassium transporters (HKTs) are suggested to mediate a substantial Na<sup>+</sup> influx in many species (Uozumi et al. 2000, Horie et al. 2001, Golldack et al. 2002, Mäser et al. 2002, Gárciadeblás et al. 2003). In rice, nine HKT homologues are identified (Gárciadeblás et al. 2003), out of which eight encode proteins with distinct transport activities, which might be expressed in various tissues and/or organs (Kader and Lindberg 2008).

### 32.6.4 BREEDING

Like all other stresses, breeding of tolerant cultivars is crucial to fight the ill effects caused by high salt concentration. Both breeding and screening germplasm for salt tolerance encounter the following limitations: (a) different phenotypic responses of plants at different growth stages, (b) different physiological mechanisms, (c) complicated genotype × environment interactions, and (d) variability of the salt-affected field in its chemical and physical soil composition (Arzani 2008). In general, two main approaches are being used to improve salt tolerance: (1) the exploitation of natural genetic variations, either through direct selection in stressful environments or through mapping QTLs and subsequent marker-assisted selection; and (2) the generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance (Yamaguchi and Blumwald 2005).

Breeding for salt tolerance has been proposed to the extent of possible crop production in sea water (Boyko 1966, Epstein and Norlyn 1977). Of various ways to tackle the salinity problem, exploitation of genetic mechanism is the most important strategy. Deliberate exploitation of genetic mechanism is mainly possible through (1) the direct use of halophytes (Malcolm 1969, Malcolm and Allen 1981, O'Leary 1994.) or choosing salt-tolerant crops as per the problem; (2) the introgression of tolerant genes from salt-tolerant genotypes (related or distant) (Flowers and Yeo 1995), and (3) the use of nonconventional approaches, such as tissue culture and molecular biology.

Despite having knowledge about a long list of halophytes and their economic potential as fodder, fuel (Malcolm 1969, Malcolm and Allen 1981, Flowers and Yeo 1995), and oilseed (O'Leary 1994), their direct use as an economic crop is still under dormancy. Among these, jojoba (though

not too salt tolerant) is a suitable crop for such areas (Shanon 1997). On the basis of our knowledge about the sensitivity of crop plants to high salt concentration, appropriate crops can be grown as per the intensity of the salt concentration in the soil. Cultivated crop plants can be classified into tolerant, intermediate, and sensitive types. Shanon (1997) has presented an elaborate review on the genetic variability of domesticated crop plants to salt stress. Barley is one of the most salt-tolerant crops; other tolerant crops are sugarbeet, cotton, canola (*Brassica* spp.), asparagus, red beet, zucchini squash, date palm, pomegranate, grape, wheat grass, bermuda grass, etc. Crop plants having an intermediate tolerance are sorghum, sunflower, safflower, sugarcane, potato, alfalfa, faba bean, almond, plum, orange, grape fruit, pea nut, chrysanthemum, carnation, etc., while the salt-sensitive group includes rice, corn, wheat, legumes, linseed, cowpea, lentil, chickpea, citrus, avocado, stone fruits, apricot, peach, blackberry, strawberry, aster, poinsettia, gladiolus, azalea, gardenia, gerbera, amaryllis, African violet, etc. The introgression of salinity tolerance has been attempted from related genera and species in some crops, such as wheat and tomato, but without success due to the absence of the right knowledge about the exact kind of traits, their genetics (Chaubey and Senadhira 1994, Winicov 1994), and difficulties in recovering the traits of agronomic value. However, intervarietal crossing has yielded successful salt-tolerant genotypes in some crops. For example, few salt-tolerant wheat varieties (KRL 1-4, Raj 3077, WH 157, and JOB 66) have been developed in India during the past few years, and are being successfully grown in salt-affected areas. Based on the problems associated with the breeding of salt-tolerant genotypes in crops, it has been suggested that it is better to select for yield rather than for salt tolerance (Richards 1992, Jain et al. 1993). Rosielle and Hamblin (1981) also suggested that selection for productivity will increase yields in both stressed and nonstressed environments. However, this strategy may not work in all agro-eco environments, for instance, in a water-logged condition, where salt-tolerant rice is the only alternative (Flowers and Yeo 1995). Therefore, the use of physiological parameters might prove a useful component of breeding through pyramiding component physiological traits at least in sensitive species (Flowers and Yeo 1995). According to Flowers and Yeo (1995), salt-tolerant genotypes can be developed through a crossing programme that maximizes recombination, followed by single seed descent and selection for resistance along with agronomic characters.

Among the novel ways of enhancing the salt tolerance of crops, the important ones proposed are the use of undifferentiated cells in tissue culture, and gene manipulation through molecular biology. Although difficulties are still present in both these methods, some success has been obtained in crops such as alfalfa (Winicov 1991), bent grass (Kuo et al. 1994), potato (Naik and Widholm 1993), and citrus (Kochba et al. 1982, Ben-Hayyim and Goffer 1989). The major problem in breeding for salinity tolerance continues to be the quantitative nature of stress tolerance and the problems associated with developing proper replicable testing environments (Arzani 2008).

## 32.7 ACID SOIL TOLERANCE

Similar to other abnormal environments, a low pH of soil also retards plant growth and development, thereby causing yield reductions. Soil acidity is a function of  $H^+$  activity in soil solution (Johanson 1988) and shows both chronological and spatial (horizontal and vertical) variation (Carver and Ownby 1995). Acid soils are phytotoxic due to a complex of nutritional disorders, which includes both deficiency (Ca, Mg, Mo) and excess (Al, Mn, H) of different nutrients (Adams 1984, Robson 1989). In this complex situation, the most damaging is the Al toxicity (Foy et al. 1978), which causes a number of disorders and may also influence water stress tolerance of crops (Goldman et al. 1989, Carver and Ownby 1995). Mn toxicity, not as important as Al toxicity, has also received attention during recent years (Foy et al. 1988, Mukhopadhyay and Sharma 1991).

Acid soils are scattered all over the world in patches with a greater proportion in tropical regions. They significantly limit crop production worldwide, because approximately 50% of the world's potentially arable soils are acidic (Kochian 2004). Hence, this is of concern to a vast population of growers. The increased awareness about the soil acidity problem and consequent yield reductions

has attracted researchers to unravel the mechanism of resistance against the acid pH of soils (Foy et al. 1988, Haug and Shi 1991, Taylor 1991, Rao et al. 1993, Carver and Ownby 1995). Aluminum toxicity, being most crucial, has attained most attention in our venture to understand the tolerance to soil acidity.

Mechanisms involved in Al tolerance are complex and could differ among species (Hanson 1991). Plants tolerate Al toxicity in two ways: (1) Al exclusion from plant tissues, especially the symplastic portion of root meristems (e.g., by chelation of Al by organic acids); and (2) internal Al detoxification, by converting Al into a harmless form (Hanson 1991, Carver and Ownby 1995, Delhaize et al. 2007). In contrast to Al, Mn tolerance seems to be largely based on an internal mechanism only. The probable reasons for this are the role of Mn as an essential element, and the biological and chemical similarities between Mn and Mg (Carver and Ownby 1995). However, recent evidence has shown that organic acids also play an important role in detoxifying Al internally and externally (Ma 2000, Ma et al. 2001, Ryan et al. 2001, Ma and Furukawa 2003). Since, both kinds and the amount of exuded OA anions contribute to the Al-detoxification capacity, the OAs have been classified as strongly (citrate, oxalate, and tartrate), moderately (malate, malonate, and salicylate), and weakly (succinate, lactate, formate, and acetate) Al-detoxifying compounds according to the stability of the Al complex (Hue et al. 1986). The enhanced exudation of citrate in response to Al stress has been reported in a number of crops, namely, common bean (Shen et al. 2002, Stass et al. 2007), maize (Pellet et al. 1995, Kollmeier et al. 2001), and soybean (Yang et al. 2000, 2001, Silva et al. 2001); the exudation of malate in wheat (*T. aestivum* L. (Delhaize et al. 1993, Pellet et al. 1997); a combination of both (citrate and malate) in *S. cereale* L. (Li et al. 2002) and triticale (x *Triticosecale* Wittmack) (Ma et al. 2000, Hayes and Ma 2003); and oxalate in buckwheat (*Fagopyrum esculentum* Moench) (Zheng et al. 1998) and taro (*Colocasia esculenta* L. Schott.) (Ma and Miyasaka 1998). These OAs are thought to complex Al within the apoplast of the root apex (Kinraide et al. 2005). Recently, it has been reported that aluminum resistance in common bean (*P. vulgaris*) involves the induction and maintenance of citrate exudation from root apexes (Rangel et al. 2009).

### 32.7.1 MORPHOPHYSIOLOGICAL TRAITS: GENETIC BASIS

There is strong correlation between soil acidity and the root growth of plants (Khaliwada et al. 1916, Hanson 1991, Bona et al. 1994). The inheritance of root length under acid soils in wheat showed polygenic control with a wide range of the degree of dominance (Bona et al. 1994). In rice, the relative root length under acidic pH showed both additive and dominance effects with a preponderance of the additive effect; the trait was partially dominant with high heritability, and one group of genes was detected (Khaliwada et al. 1916). In soybean, along with thicker roots, increased seed weight was also found as an associated response with selection for seedling tolerance to acidity (Hanson and Kamprath 1979, Hanson 1991). For Mn, a common gene system in both the root and shoot of wheat has been suggested (Burke et al. 1990).

Among physiological mechanisms countering low pH effect, the exudation of organic acids by plant roots is the most acceptable mechanism of external tolerance to Al toxicity (Delhaize et al. 1993). Al tolerance is also reported to be associated with greater efficiency of phosphate uptake (Foy et al. 1978, de Miranda and Rowell 1990) and cation exchange capacity (Blamey et al. 1990). Though there are indications of variation for these traits (Blamey et al. 1990, de Miranda and Rowell 1990, Delhaize et al. 1993), genetics of these traits has not been elucidated.

### 32.7.2 GENETICS

Substantial genetic variation for tolerance to acid soil has been reported in different crops, such as wheat (Foy et al. 1965, Lafever et al. 1977, Berzonsky 1992, Gustafson and Ross 1990, Carver and Ownby 1995), rice (Sivaguru et al. 1992), maize (Rhue et al. 1978, Miranda et al. 1984, Kasim et al. 1990), soybean (Hanson 1991), etc. Genetic studies on acid tolerance in crops indicate both

qualitative and quantitative inheritance. Monogenic control was reported in wheat (Foy et al. 1965, Kerridge and Kronstad 1968, Lafever et al. 1977, Larkin 1987, Wheeler et al. 1992) and maize (Rhue et al. 1978, Miranda et al. 1984). Two dominant genes have been shown to be responsible for Al tolerance in the wheat variety Atlas 66 (Camargo 1981), while several genes were found in a relatively less tolerant line Chinese Spring (Carver and Ownby 1995). Resistance to Al toxicity may be different at seedling and adult plant stages (Carver and Ownby 1995).

Quantitative inheritance for tolerance in acid soils is reported in wheat (Aniol 1984, Bona et al. 1994), rice (Khaliwada et al. 1916), maize (Magnavaca et al. 1987, Sawazaki and Furlani 1987, Lima et al. 1992, Pandey and Gardner 1992, Duque-Vargas et al. 1994), and soybean (Hanson 1991). Al tolerance was dominant over sensitivity, but, at the same time, it showed a greater role of the additive gene effect (Aniol 1984, Bona et al. 1994). Allelic variation for genes controlling Al tolerance has been noted in wheat (Lafever and Campbell 1978), barley (Minella and Sorrells 1992), and maize (Rhue et al. 1978). The change in the direction of dominance with a change in Al toxicity has been reported in wheat (Camargo 1981, Campbell and Lafever 1981, Bona et al. 1994) and barley (Minella and Sorrells 1992), which might be due to the differential expression of tolerant genes at varying levels of Al concentrations. In rice, both general combining ability (GCA) and specific combining ability (SCA) were important for Al toxicity (Khaliwada et al. 1916), but GCA was more prevalent. Reciprocal effects were also noticed (Khaliwada et al. 1916). Similarly, in maize, both additive and dominance genetic variations were reported for yield under acid soils (Magnavaca et al. 1987, Sawazaki and Furlani 1987, Lima et al. 1992, Pandey and Gardner 1992, Duque-Vargas et al. 1994).

In wheat, hexaploid (AABBDD) wheat is more tolerant than tetraploid or diploid. The tolerance of the D genome is maximum followed by A and B genomes, respectively. The R genome of rye (*S. cereale*) possesses even greater tolerance than the D genome of wheat. The N genome of wheat (*T. ventricosum*) also possesses acid soil tolerance. Although the genes associated with Al tolerance are present in all the three genomes of wheat, the most important locations are 2DL and 4DL (Takagi et al. 1983, Aniol and Gustafson 1984, Aniol 1990). In rye, Al tolerance genes are located on chromosomes 3R, 4R, and 6R (Aniol and Gustafson 1984), but show reduced tolerance when transferred to wheat, probably due to their suppression by unknown genes of wheat (Aniol and Gustafson 1984).

A wide genetic variation for Mn tolerance has also been recorded in wheat (Foy et al. 1988, Macfie et al. 1989) with the suggestion that only few genes are involved in the Mn tolerance (Foy et al. 1988). It has been suggested that the inheritance of tolerance to Al and that to Mn are independent and different genes may be involved (Neenan 1960, Foy et al. 1973, Burke et al. 1990, Fisher and Scott 1993), but there might be genes for the coregulation of their inheritance (Carver and Ownby 1995).

### 32.7.3 GENE EXPRESSION

Plants' ability to tolerate soil acidity is associated with a syndrome of cellular and molecular activities. A large number of genes take part in the whole operation, synthesizing different proteins. The idea that plants develop Al tolerance through the synthesis of proteins capable of inactivating Al (Aniol 1984) has now grown to a near reality with the identification of several proteins showing increased synthesis in response to Al (Slaski 1990, Horst et al. 1991, Cruz-Ortega and Ownby 1993, Snowden and Gardner 1993). A protein named RMP51 (51 kDa) has been reported to occur in the roots of an Al-tolerant cultivar of wheat; this protein has shown insensitivity to Mn, Cu, and heat stress (Basu et al. 1994). However, none of the known proteins can be said to be the product of a gene conferring Al tolerance (Carver and Ownby 1995).

### 32.7.4 BREEDING

In view of the increasing food demand and shrinking land resources, the need for a genetic improvement of crops for their tolerance to acid soils is beyond question. This might be a case of necessity



rather than economics, though might prove useful in the long run. It is true that genetic tolerance does not correct the problem of soil acidity and only postpones the need to take corrective actions (Carver and Ownby 1995), but a judicious crop cultivation may have a beneficial effect on the soil in a variety of ways. Tolerance to acid soils varies from crop to crop and genotype to genotype. For example, rye is more tolerant than common wheat, and common wheat is more tolerant than barley. In wheat, a number of Al-tolerant genotypes have been identified, for example, Atlas 66. Genotypes of Brazilian origin show high tolerance to Al toxicity (Carver and Ownby 1995).

Breeding for acid soil tolerance has gained momentum with the development of reliable screening techniques. Both laboratory and field screening methods are used. The most common screening medium for Al and Mn tolerance is solution culture, which is a nondestructive measurement of tolerance. During screening, the tolerance is generally measured based on the damage to root/shoot (Konzak et al. 1997) and the degree of severity following exposure to Al (Aniol 1984).

The presence of dominance for acid soil tolerance has enabled breeders to use the backcross method of breeding for improving acid soil tolerance. A successful example is the transfer of a major gene for Al tolerance from the Carazinho variety of wheat to Egret (Fisher and Scott 1987) in Australia. It has been suggested that an early generation selection may be beneficial in breeding for Al tolerance in rice, and the pedigree method may also be used (Khatriwada et al. 1916). Recurrent selection has also been suggested as an alternative method to exploit the additive gene action related to Al tolerance in wheat (Carver et al. 1988, Minella and Sorrells 1992) and maize (Magnavaca et al. 1987, Lima et al. 1992, Granados et al. 1993). In the CIMMYT breeding programme, which utilizes a shuttle-breeding program, selection for acid tolerance is generally done following the evaluation of genotypes for yield and quality (Rajaram et al. 1986).

Interspecific hybridization is also a possible way of improving acid soil tolerance in crops. For example, tetraploid hybrids of *T. aestivum* × *T. ventricosum* (Maan 1987) were developed for meeting the same objective. Molecular biology, though under investigation (Putrill et al. 1991, Snowden and Gardner 1993, Basu et al. 1994), is still to play a role in breeding for tolerance to acid soils.

## 32.8 CONCLUSIONS

The increasing pressure on our natural resources including land is being realized by all. As our land area cannot be increased, the only hope of extending the cultivable area is through a greater utilization of the so-called “unfit areas,” which suffer from one or the other problem. A big chunk of this area suffers from severe abiotic stresses. However, this does not mean that abiotic stresses do not occur in other areas. In fact, abiotic stresses are prevalent in almost all cultivable lands, though in variable intensities and durations. It is common to see our crops suffering or fighting with abiotic stresses such as abnormal levels of water, temperature, and soil pH. When our crop plants suffer, we also suffer, directly or indirectly.

Despite abiotic stresses being so vivid and important for crop production, tolerance mechanisms are not understood the way they should be and efforts to enhance the tolerance of crops are still far from satisfactory. Of various reasons for this slow progress, the most important ones are the absence of clear-cut traits conferring tolerance and the complexity caused by the simultaneous occurrence of more than one stress in variable intensities and durations. However, efforts of various researchers have succeeded in correlating some morphophysiological traits with concerned stresses along with the genetic mechanism involved. Molecular genetics is also contributing in a slow but sure manner to expand our knowledge in this direction. Several varieties have been released in different countries for meeting the challenge posed by abiotic stresses. Even a man-made crop like triticale was created to occupy marginal lands with a high stress pressure. Though abiotic stresses can be tackled better by an integrated approach, genetic manipulation should remain on top of this strategy. Growing knowledge of molecular genetics appears to be the key of the future.

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# 33 Genetic Improvement of Cold Hardiness in Bermudagrass

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## 33.1 INTRODUCTION

The revised taxonomy of the genus *Cynodon* L. C. Rich. consisting of eight species (Harlan et al.1970a) has been widely adopted (Clayton and Renvoize 1989). “Bermudagrass” is a common name, widely used for several species of plants in the genus *Cynodon*, except for three large, robust, non-rhizomatous species originated in East Africa. Harlan (1970) noted that “star grass” is a proper name for the large East African species including *C. aethiopicus* Clayton et Harlan, *C. nlemfuensis* Vanderyst, and *C. plectostachyus* Pilger. *Cynodon dactylon* (L.) Pers. var. *dactylon* is widely known as “common bermudagrass” and *C. transvaalensis* Burtt-Davy as “African bermudagrass.” From the viewpoints of agriculture, economics, and environmental protection, bermudagrass is extremely important due to its extensive worldwide use for forage, turf, and soil and water protection. More recently, bermudagrass has been proposed as a potential cellulosic biofuel crop (Anderson et al. 2008).

Bermudagrass is a warm-season, sod-forming, perennial grass that utilizes the C<sub>4</sub> photosynthetic carbon fixation pathway (Krans et al. 1979). The optimum growth of bermudagrass plants occurs between 27°C and 35°C (Turgeon 2008). Cold hardiness is a major limiting factor determining the adaptation range of bermudagrass as its use extends beyond tropical and subtropical regions, the primary adaptation area for bermudagrass. Use at higher latitudes results in a greater risk of winter-kill (Taliaferro et al. 2004a). For example, in the United States, bermudagrass has been used in the transition zone between the primary cool-season turfgrass belt in the northern states and primary warm-season turfgrass belt in the southern states. Although not well defined, the climatically variable transition zone is within the range of the U.S. Department of Agriculture (USDA) plant hardiness zones 5 through 7. Some harsh winters in the transition zone are so severe that bermudagrass stands can be extensively damaged or killed. The costs associated with extended loss of use and investments in reestablishment of bermudagrass are substantial (Anderson et al. 2005). Fortunately, bermudagrass freeze tolerance is a heritable trait, cold hardy bermudagrass germplasm exists in

nature, and plant breeding is able to genetically incorporate this desirable trait into improved cultivars. Development and release of cultivars improved in freeze tolerance reduces the winterkill risk when bermudagrass is used in the transition zone and extends its use to higher latitudes. This chapter addresses the topics of economic significance, germplasm variability, and distribution related to cold hardiness, development of cultivars with improved cold hardiness for forage and turf use, evaluation and determination of freeze tolerance, related physiological and molecular mechanisms, and future potential to continue the improvement of bermudagrass freeze tolerance.

### **33.2 IMPORTANCE AND ECONOMIC USES OF BERMUDAGRASS**

Bermudagrass has probably been grazed by herbivorous animals, used for turf, soil protection, or similar purposes for many hundred years or even longer in the geographic and climatic regions where environment factors are conducive for its growth. These past uses were probably sporadic and small in scale, with no documented records indicating any economic significance until recently. Bermudagrass, becoming a major grass cultivated in monoculture on millions of hectares for forage and turf is a phenomenon developed in the United States over the last two centuries, especially after improved cultivars were bred and released from organized breeding programs in the last six decades (Taliaferro et al. 2004b). Subsequently, improved forage and turf bermudagrass cultivars have received larger applications in many other countries in the world.

#### **33.2.1 FORAGE GRASS**

Hanna (2007) noted bermudagrass is one of the most widespread and heavily used grasses. Many inherent attributes make bermudagrass an important and popular forage grass. Desirable characteristics include high yield capability, perennial growth habit, drought tolerance, and tolerance to close defoliation from animal grazing (Taliaferro 2005). Bermudagrass has few devastating insect or disease problems. The aggressive spreading capability of bermudagrass reduces weed encroachment into its stands. Propagation of bermudagrass is easily realized by sowing seed or planting vegetative propagules. Bermudagrass responds well to management practices such as sufficient water availability, frequent cutting, and balanced fertilization of nitrogen, phosphorus, and potassium. Bermudagrass is well adapted to grazing because of its extensive stolons and rhizomes. New leaf growth following repeated defoliation is nutritious and digestible, although mature bermudagrass is low in forage quality. Bermudagrass forage cultivars improved in one or more desirable traits, such as forage yield, digestibility, cold hardiness, and drought tolerance, have contributed substantially to the rise in prominence and establishment of the species as a major warm-season forage grass in the southern United States. Bermudagrass, along with other warm-season perennial grasses, forms improved pastures and forage systems, which provide a feed base for an immense animal husbandry industry in the southern U.S. states and many other warm climate regions in the world. For example, Bouton (2007) estimated the economic value of the forage systems for beef cattle and calf production in 14 southeastern U.S. states (Oklahoma, Texas, Louisiana, Arkansas, Missouri, Tennessee, Mississippi, Alabama, Georgia, Florida, South Carolina, North Carolina, Kentucky, and Virginia) is approximately US \$11.6 billion annually. Bermudagrass is a major component of the forage systems in the southeastern United States with an estimated 10–12 million hectares devoted to animal herbage (Taliaferro et al. 2004b).

#### **33.2.2 TURFGRASS**

Bermudagrass is the most widely used warm-season turfgrass in the world (Shearman 2006). In addition to the many desirable traits of bermudagrass discussed above, its dense sod-forming ability, tolerance to close mowing, long-lived perennial nature, and wide adaptation make bermudagrass an attractive turfgrass (Taliaferro et al. 2004a). Beard (1973) noted that bermudagrass is a good choice

for many turf areas if the environment is suitable for its growth. He indicated the heat and drought tolerance of bermudagrass is excellent, but cold hardiness is poor in general. In warmer temperate, subtropical, and tropical regions, improved turf bermudagrass forms a very dense and uniform turf of high quality, making it suitable for establishment of a myriad of turf forms. Bermudagrass has excellent wear tolerance and recuperative capacity, which are important for athletic turf fields. Turf bermudagrass is extensively used on residential lawns, parks, institutional grounds, athletic fields, and roadside areas. It is also widely deployed to establish vegetations on golf courses, including fairways, tee boxes, putting greens and rough zones. In the United States and several other countries, the sod industry produces a large amount of turf bermudagrass sod, which can immediately cover the areas to be established. Establishing turf using bermudagrass seed is another widely used propagation means. Bermudagrass turf area in the United States is huge, though it is difficult to pinpoint the size. There is a total of 23 million hectares of managed turfgrass in the United States (Shearman 2006), with bermudagrass being the predominant warm-season turfgrass.

### 33.3 GERMLASM DISTRIBUTION: ENDEMIC AND COSMOPOLITAN SPECIES

*Cynodon* species vary widely in germplasm distribution, cold hardiness, and agronomic value for breeding forage and turf cultivars. *Cynodon dactylon* is the only ubiquitous and cosmopolitan taxon, and consequently it contains the greatest genetic diversity, the widest geographic distribution, and the greatest economic value for breeding forage and turf cultivars within the genus (Wu 2010). *Cynodon transvaalensis* is an endemic species in South Africa that has been very important for the development of clonal hybrid turf cultivars in hybridizations with *C. dactylon* (Burton 1965, 1991). *Cynodon nlemfuensis* is endemic in East Africa and the most promising and valuable stargrass species used in forage bermudagrass breeding (Burton 1972, Burton et al. 1993). Selected *C. nlemfuensis* plants have been used to cross with *C. dactylon* genotypes in the development of interspecific forage hybrid cultivars improved in dry matter digestibility and/or biomass yield since *C. nlemfuensis* is genetically compatible with *C. dactylon*. However, germplasm distribution patterns and cold hardiness of the three species are distinct. It appears that natural distribution and cold hardiness in *Cynodon* species are closely related. Harlan et al. (1970a) noted there is little cold hardiness in *C. nlemfuensis*, *C. aethiopicus*, *C. arcuatus*, *C. barberi*, and *C. plectostachyus*. Those five species all have tropical and subtropical origins although their respective distribution patterns are different (Harlan et al. 1970b). *Cynodon incompletus* Nees, a species endemic to South Africa, is cold hardy but has little value as a forage grass (Harlan 1970). Germplasm of the latter species has not been used in turf bermudagrass breeding programs as well. Therefore, germplasm within *C. dactylon* appears to be the sole or major source to provide cold resistance genes for the development of cold hardy forage bermudagrass cultivars, while development of cold hardy turf bermudagrass cultivars can use germplasm from both *C. dactylon* and *C. transvaalensis*.

The species *C. dactylon* consists of six botanical varieties: *dactylon*, *afghanicus* Harlan et de Wet, *aridus* Harlan et de Wet, *coursii* Harlan et de Wet, *elegans* Rendle, and *polevansii* (Stent) Harlan et de Wet (Harlan et al. 1970a). Variety *dactylon* is the only one which has a truly cosmopolitan geographic distribution in the world, being one of the most widely distributed of all plants (Harlan et al. 1970a). Its winter hardiness varies from none to very hardy. The taxon contains plants varying enormously in size from small statured varieties which have been used as turf grasses and in the development of modern turf cultivars, to large plants used as pasture and forage grasses and in breeding improved forage cultivars. Variety *dactylon* plants are distributed to about 45° south latitude in South America and 53° north latitude in northern Europe (Harlan and de Wet 1969). Harlan and de Wet (1969) indicated that var. *dactylon* is found in every country in Africa, in North, Central, and South America, Australia, New Zealand, and every country in Asia except Mongolia. In Europe, var. *dactylon* is ubiquitous and found in all countries except the most northern regions. In Nepal and southwest China, var. *dactylon* is found growing about 3000m above sea level. Variety *dactylon* was introduced in the United States in or before 1751 (Taliaferro et al. 2004b).



Currently, it is found in all southern and central states, and some northern states, such as New Hampshire, New York, Michigan, Montana, Idaho, and Washington in the United States, and British Columbia in Canada (USDA NRCS 2009). Taliaferro et al. (2004b) indicated var. *dactylon* occurs in greatest abundance in tropical and subtropical environments. Variety *dactylon* is less abundant at higher latitudes. Natural distribution to higher latitudes is indicative of cold hardy germplasm in var. *dactylon*.

Harlan and de Wet (1969) proposed three races within var. *dactylon* on the basis of adaptation, distribution, and morphology. Tropical race plants are widely distributed in pantropical areas and 20 cm or less in stature. Plants in the tropical race have little winter hardiness. The temperate race resembles the tropical race in morphology, but is winter hardy. Plants of the temperate race are distributed in northern Europe, northern United States, and northern China. Seleucidus race plants are very coarse in leaf texture and distributed in Afghanistan, Turkey, Iran, Turkmenistan, Uzbekistan, Greece, Bulgaria, and Yugoslavia. Moving north to Europe, seleucidus plants become smaller and merge with the temperate race (Harlan and de Wet 1969). The seleucidus race gets its name because most typical plants of the race are distributed in the region from Turkey to Afghanistan once occupied by the Seleucid empire (Harlan and de Wet 1969). Harlan and de Wet (1969) have shown that plants typical of the seleucidus race are very cold hardy. The relative cold hardiness assessment of the above-mentioned three races was made in field observations by Harlan and colleagues in 1960s. No quantitative data are available to reveal cold hardiness variability of the three races.

Variety *aridus* is naturally distributed in arid regions in South Africa, East African countries, Egypt, Israel, Arabia, India, Sri Lanka, and Myanmar. Plant size ranges from small in India to large in South Africa. Introduced var. *aridus* plants called “Giant bermudagrass” have been grown in Arizona. Harlan and de Wet (1969) indicated var. *aridus* plants in their collection from South Africa, India, Israel, and the Near East had no winter hardiness in field plots tested in Stillwater, Oklahoma. However, they can survive winters by producing deep rhizomes that escape freezing temperatures. Cooper and Burton (1965) reported that giant bermudagrass produces no more forage than common bermudagrass, which produces much less forage than the released cultivar “Coastal” bermudagrass in Georgia. Giant bermudagrass is inferior to common bermudagrass as a turf grass. However, from the evolutionary point of view, var. *aridus* may play an extremely important role as the progenitor of var. *dactylon*, which likely is an autotetraploid (Harlan and de Wet 1969).

Variety *afghanicus* is so named because plants of this variety are only found in Afghanistan (Harlan and de Wet 1969). The endemic variety plants are tall and have long stolons. Plants of this variety normally are found growing in lowland steppes and along irrigation ditches. Although var. *afghanicus* has no rhizomes in diploid plants and has short rhizome-like structures in tetraploid plants, they have good winter hardiness since field-grown plants can survive winters at Stillwater, Oklahoma (Harlan and de Wet 1969, Harlan et al. 1970a). Harlan and de Wet (1969) believed the var. *dactylon* seleucidus race is derived from “the infusion of var. *afghanicus* germplasm into the temperate race of var. *dactylon*.”

*Cynodon transvaalensis* is endemic to South Africa (de Wet and Harlan 1970, 1971, Harlan et al. 1970a). Plants of *C. transvaalensis* are unique and distinct from all other plant species including other *Cynodon* species. African bermudagrass plants are small in size, turf forming, and have purple, fine wirelike stolons. Other morphological characteristics of African bermudagrass include its slender leaf blade (<2 mm in width), yellowish-green color, and erect habit due to narrow angles between leaves and shoots (de Wet and Harlan 1971). Each inflorescence is composed of two to three (rarely four) short racemes. Plants in the original areas are often found in damp habitats around water sources and along stream banks. The species is distributed in a region from southwestern Transvaal, Orange Free State, to the northern part of central Cape Province in South Africa, approximately between 25° and 30° south latitudes (Harlan et al. 1970a). However, African bermudagrass plants have good winter hardiness at Stillwater, Oklahoma (36° north latitude). Taliaferro (2003) noted some African bermudagrass plants have demonstrated ample cold hardiness to survive winters at 39° north latitude in the United States. Harlan et al. (1970b) indicated

the turf-forming, cold hardy species has far more winter hardiness than needed in the original habitats. This may suggest African bermudagrass is a relic of ancient bermudagrass, which may have survived the Pleistocene climates of South Africa (Harlan et al. 1970b).

### 33.4 DEVELOPMENT OF COLD HARDY FORAGE AND TURF CULTIVARS

Breeding superior bermudagrass cultivars for forage and turf has achieved unprecedented success in the last seven decades. The bermudagrass breeding program of Dr. Glenn Burton (USDA-ARS geneticist) began to breed forage cultivars in 1936 and subsequently added turf bermudagrass breeding into his program in 1946 at the Coastal Plains Experiment Station, Tifton, Georgia (Burton 1991). Harlan (1970) assessed that the development of improved forage bermudagrass cultivars including “Coastal” profoundly impacted the livestock industry in the southern United States. Recent reviews by Shearman (2006) and Nelson and Burns (2006) further documented the enormous contributions of improved forage and turf bermudagrass cultivars, along with other important grass species, to the modern turf industry and animal husbandry, not only in the United States, but in the world as well. Breeding methods used to develop forage and turf cultivars in bermudagrass primarily encompass intra- and interspecific hybridizations to produce segregating progeny populations from which clonal hybrid cultivars are selected and developed, and cyclic population improvement used in cross-pollinated, self-incompatible perennial grasses to develop seeded cultivars, often referred to as synthetic cultivars or simply synthetics. Taliaferro (2003) pointed out that early cultivars of bermudagrass prior to the 1940s were selections from naturally occurring variation. Released bermudagrass cultivars have been improved in individual traits or combinations of several traits. The improved traits include, but are not limited to biomass yield, digestibility, and animal body gain for forage use, and turf quality and related traits, such as texture, color, density, and uniformity for turf use, and resistance to abiotic and biotic stresses for both types. Cold hardiness is the most important adaptation trait facilitating the extension of bermudagrass beyond tropical and subtropical environments to colder areas. In the transition zone of the United States, improving cold hardiness will substantially increase commercial value of bermudagrass cultivars (Wu and Taliaferro 2009). Increasing cold hardiness to enhance bermudagrass adaptability in the transition zone has been one of the main breeding objectives since the inception of the Oklahoma State University bermudagrass breeding program initiated by Dr. Charles Taliaferro in 1968. The selected cultivars described below as milestone achievements are presented as representative of the progress made in breeding cold hardy forage and turf bermudagrass. Since elevated cold hardiness is an adaptation trait which should reduce winterkill risk in the northern part of the bermudagrass belt, combination of increased cold hardiness and improved performance traits has been the key in new bermudagrass cultivar development in the U.S. transition zone.

#### 33.4.1 FORAGE BERMUDAGRASS CULTIVARS WITH IMPROVED COLD HARDINESS

“Coastal” bermudagrass is an  $F_1$  hybrid clonal cultivar derived from a cross between a clonal female parent discovered in an old cotton patch near Tifton, Georgia and an introduced male parent from South Africa (Burton 1954). The hybrid cultivar was created by Burton and released by the Georgia Coastal Plain Experiment Station and the Division of Forage Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering in 1943 (Myers 1951). Coastal is highly productive and produced up to two times the yield of naturalized common bermudagrass. Coastal has proven to be widely adapted in the southern part of the U.S. bermudagrass belt. It has cold tolerance to be persistent in regions which are below approximately 33° to 34° north latitudes or a line connecting the southern borders of Oklahoma, Tennessee, and North Carolina in the United States (Burton and Hanna 1985). “Midland” bermudagrass is an  $F_1$  hybrid clonal cultivar derived from a cross between Coastal (male parent) and a winter hardy female parent collected in Indiana (Harlan et al. 1954). The cross was

made by Burton in 1942 at Tifton, Georgia (Hein 1953). It was tested in Georgia, Oklahoma, and other states in the northern range of the U.S. bermudagrass belt. Midland yields more forage than common strains in fertile soil. Midland has been grown successfully where Coastal winter-killed (Burton and Hanna 1985). Midland was jointly released in 1953 by USDA ARS and the Oklahoma Agricultural Experiment Station (OAES).

“Greenfield” bermudagrass is a clonal cultivar selected from many common bermudagrass strains grown on the OAES Agronomy Farm at Stillwater (Elder 1955). It was released by OAES in 1954. Greenfield has a faster establishment rate than Coastal and Midland. It produced forage favorably compared to Midland bermudagrass on moderate to low fertility soils (Elder 1955). Greenfield bermudagrass is cold hardy, makes greenup early in spring, and is persistent in eastern Oklahoma which receives 30 in. or more rainfall per year. It is a grazing-type cultivar which has been widely used in eastern Oklahoma as a pasture grass.

“Hardie” bermudagrass is a clonal cultivar released by OAES in 1974 (Taliaferro and Richardson 1980). Hardie was selected from  $F_1$  hybrids derived from crosses between a collection from Turkey (*C. dactylon* var. *dactylon*) and a progeny of two variants of PI 223248 (*C. dactylon* var. *afghanicus*), a collection from Afghanistan. The cultivar produced 6% more dry matter, which was 6% more digestible than Midland bermudagrass. Hardie is cold hardy, but susceptible to leaf disease (Taliaferro et al. 2004b).

“Tifton 44” bermudagrass is an  $F_1$  hybrid clonal forage cultivar released by USDA-ARS and Georgia Agricultural Experiment Station in 1978. Tifton 44 was selected for cold hardiness from several thousand progenies of a cross between Coastal and an accession from Berlin (Burton and Monson 1978). The Berlin bermudagrass collected by Burton in 1966 is sufficiently cold hardy to survive winters in Michigan. Tifton 44 produces more forage and has better disease resistance than Midland. Adaptation of Tifton 44, including winter hardiness, is similar to or a little less than Midland. Tifton 44 bermudagrass forage is 5%–6% more digestible than Coastal forage.

“Midland 99” bermudagrass is an  $F_1$  hybrid from a cross between PI 269370 (*C. dactylon* var. *afghanicus*) and PI 292143 (*C. nlemfuensis* var. *robustus*), and a hybrid between A12156 and A10978b-4 (Taliaferro et al. 2002). The latter two plants belong to *C. dactylon* var. *dactylon* and were collected in Oklahoma. Midland 99 was jointly released by the Oklahoma, Kansas, Arkansas, and Missouri Agricultural Experiment Stations, the Samuel Roberts Noble Foundation, and the USDA-ARS in 1999. Midland 99 is higher both in forage yield and forage quality than Midland and Greenfield, and the same or better than Tifton 44 (Taliaferro et al. 2004b). It has good stand persistence with broad adaptation. Freeze tolerance of Midland 99 is similar to Greenfield, and slightly better than Midland and Tifton 44.

“Goodwell” bermudagrass is an  $F_1$  hybrid clonal cultivar released by OAES in 2007 (Wu and Taliaferro 2009). Goodwell was derived from the cross 74X 12–11  $\times$  74X 12–12 made in 1984. The 74X 12–11 and 74X 12–12 parents were  $F_1$  hybrids from the crosses A9959  $\times$  SS-28 and SS-16  $\times$  Colorado, respectively. The SS-28 and SS-16 parents were  $F_1$  hybrids from the crosses S-16  $\times$  A9945 and S-16  $\times$  9958, respectively. The S-16 parent was an  $F_1$  hybrid from the cross A8800  $\times$  A10421. A9945 (PI 206427), A9958 (PI 251809), A9959 (PI 253302), Colorado, and A8800 (PI 269370) are clonal accessions from Turkey, Italy, Yugoslavia, Colorado, and Afghanistan, respectively. Biomass performance of Goodwell relative to standard varieties including Midland, Midland 99, Ozark, Hardie, Guymon, Tifton 44, Greenfield, Wrangler, Quickstand, and World Feeder, has been the best in irrigated tests (~6 acre inches/month during the growing season) at Goodwell, Oklahoma. Goodwell bermudagrass is cold hardy and has good adaptation in the upper portions of U.S. southern states.

“Guymon” bermudagrass is the first seed-propagated cultivar with significantly improved cold hardiness over common seed-propagated bermudagrass produced in Arizona and California (Wu and Taliaferro 2009). Guymon was released by the OAES and the USDA-ARS in 1982 (Taliaferro et al. 1983). Guymon bermudagrass may be used for both forage and turf purposes. Guymon was the progeny of crosses of two winter-hardy common bermudagrass accessions with one from

Yugoslavia and another from Guymon, Oklahoma. Seed has been produced by interplanting two parental plants in alternating rows. Guymon is adapted to the upper south, surviving up to 39° north latitude in Kansas (Taliaferro et al. 2004b), while Arizona common bermudagrass would sustain serious winterkill in the region. Annual dry matter yields of Guymon are 25%–40% less than those of clonal hybrid cultivars such as Midland and Hardie.

“Wrangler” bermudagrass is a newer seed-propagated, cold hardy cultivar (Taliaferro et al. 2004b). Wrangler was released by Johnston Seed Company, Enid, Oklahoma in 1999. Wrangler has similar adaptation and yield performance as Guymon, but has approximately twice the seed yield of the latter cultivar (Wu and Taliaferro 2009). This cold hardy bermudagrass has been widely used in the U.S. transition zone.

### 33.4.2 TURF BERMUDAGRASS CULTIVARS WITH IMPROVED COLD HARDINESS

“U-3” bermudagrass (*C. dactylon*) is clonally propagated and was selected from a collection of fine bermudagrass strains from golf greens in the early 1930s by D. Lester Hall at Savannah, Georgia (Hanson 1972). The cultivar was distributed in 1946–1947 by the U.S. Golf Association and Plant Science Research Division of Agricultural Research Service (ARS). U-3 is moderately fine-leaved, cold hardy, and fast establishing. The cultivar had wide use in the southern and transition zone states, but was gradually replaced by “Tifway” and other newer improved cultivars. However, U-3 is still used in the transition zone states, including Oklahoma, due to its cold hardiness and adaptation (Anderson et al. 2001).

“Tifgreen” bermudagrass is a vegetatively propagated turf industry standard cultivar, which has been used extensively on putting greens. Tifgreen is an  $F_1$  hybrid between a fine-textured common bermudagrass and a *C. transvaalensis* plant. It was created by G. Burton in 1951 and released cooperatively by the Georgia Coastal Plain Experiment Station and the USDA ARS in 1956 (Hein 1961). Tifgreen has very fine leaf blades, produces dense turf, and tolerates low mowing. Its best adaptation region is in the lower south in the United States.

“Tifway” bermudagrass has been a turf industry standard cultivar since its release in 1960 by USDA-ARS and Georgia Coastal Plain Experiment Station (Burton 1966). Tifway is a chance hybrid between a *C. transvaalensis* female parent and a putative *C. dactylon* male parent obtained from a *C. transvaalensis* seed packet received from D. Meredith, Johannesburg, Union of South Africa in 1954 (Alderson and Sharp 1994). The cultivar represents a combination of high disease resistance, high turf quality including dark green color and medium-fine texture, and excellent fall color retention. It is well adapted in the southern United States.

“Midway” bermudagrass is an  $F_1$  hybrid between *C. transvaalensis* and *C. dactylon* selected by R.A. Keen and released in 1965 by the Kansas Agricultural Experiment Station (Alderson and Sharp 1994). Midway is a clonally propagated cultivar with medium leaf blade texture and improved winter hardiness over U-3.

“Midiron” bermudagrass, an  $F_1$  hybrid between *C. transvaalensis* and *C. dactylon*, was selected by R.A. Keen at Manhattan, Kansas and released in 1971 by the Kansas Agricultural Experiment Station (Alderson and Sharp 1994). Midiron has relatively dark green color, but loses green color and turns brown early in the fall. This clonally propagated cultivar is medium to coarse in leaf texture. One outstanding feature of Midiron is its cold hardiness which exceeds U-3.

“Midfield” bermudagrass is a clonal cultivar derived from a cross of a *C. dactylon* plant from Hays, Kansas, and a *C. transvaalensis* genotype (Pair et al. 1994a). Midfield was selected by the Kansas Agricultural Experiment Station and jointly released by the Kansas and Oklahoma Agricultural Experiment Stations in 1991. Midfield has finer texture and denser sod than Midiron, but coarser texture and less sod density than Midlawn. Midfield has dark green color and relatively high turf quality. Cold hardiness of Midfield is slightly better than Midlawn, but similar to Midiron.

“Midlawn” bermudagrass is a natural interspecific hybrid between a *C. dactylon* plant collected on the Michigan State University campus as the maternal parent and a *C. transvaalensis* selection

maintained at the Hays Branch Experiment Station, Hays, Kansas as the paternal parent (Pair et al. 1994b). Midlawn was released cooperatively by the Kansas and Oklahoma Agricultural Experiment Stations in 1991. Midlawn is cold hardy and has early spring greenup, but its level of cold tolerance may be inferior to that of Midiron. Midlawn is finer in texture and has greater sod density than Midiron. Midlawn has good tolerance to spring dead spot disease, but grows slower than some aggressive bermudagrass cultivars.

“Tifsport” bermudagrass (*C. dactylon* x *C. transvaalensis*) was registered as “Tift 94” (Hanna et al. 1997). Tifsport was cooperatively released by the USDA-ARS and the University of Georgia Coastal Plain Experiment Station in 1995. Tifsport is a mutation selected from Midiron plants treated with 80 Gy of Cobalt 60 gamma radiation. Field experiments indicated Tifsport turf quality was superior to Midiron. It is fine textured and has sufficient cold hardiness to grow in the northern part of the U.S. bermudagrass belt, including Stillwater, Oklahoma, and Lexington, Kentucky.

“Quickstand” bermudagrass (*C. dactylon*) is a clonal cultivar jointly released by the USDA-NRCS, USDA-ARS, and the University of Kentucky in 1993 (Phillips et al. 1997). Quickstand has a desirable combination of traits including winter hardiness, quick establishment rate, and a degree of resistance to spring dead spot disease. It is well adapted in the U.S. transition zone for turf and grazing purposes. Leaf texture of Quickstand bermudagrass is medium.

“Patriot” bermudagrass is a newer vegetatively propagated F<sub>1</sub> hybrid released by OAES in 2002. It is an improved interspecific turf cultivar derived from a cross made in 1992 between the hexaploid *C. dactylon* “Tifton 10” and a *C. transvaalensis* selection (Taliaferro et al. 2004c). Contrary to many interspecific clonal turf cultivars having 27 somatic chromosomes, Patriot is a tetraploid having 36 chromosomes. It combines unique dark green color, high turf quality, good sod strength, and improved cold hardiness. Patriot is well adapted to the U.S. transition zone.

“Cheyenne” bermudagrass (*C. dactylon*) is a seeded turf cultivar developed and released in 1990 by Jacklin Seed Company (Samudio and Brede 1998). Cheyenne is a synthetic cultivar produced from inter-pollinating five parent plants grown in alternating rows. The five parent plants were selected from a spaced nursery of 600 progeny of a cross of two fertile, self-incompatible clones, one from an old turf in the Columbia River Basin area in the state of Washington and the other from an open-pollinated progeny of PI 253302 originated in Yugoslavia. Cheyenne has improved turf quality over Arizona Common. Cheyenne has better spring ground coverage than Arizona Common and better winter color retention than Guymon.

“Yukon” bermudagrass (*C. dactylon*) is a seed-producing, turf-type, synthetic cultivar released in 1997 by OAES (Taliaferro et al. 2003). The synthetic bermudagrass was produced by the intercrossing of six selected clonal parents that were planted in alternating rows. The parent plants were selected from a broad genetic base breeding population with two cycles of phenotypic selections for increased fertility and finer texture. The base breeding population was formed with winter hardy germplasm accessions. Yukon exhibited exceptional cold tolerance and higher turf quality over many seeded turf bermudagrass cultivars in various tests, including the National Bermudagrass Test of the National Turfgrass Evaluation Program (NTEP). Improved cold hardiness of Yukon allows its use in the U.S. transition zone for various turf purposes.

“Riviera” bermudagrass is a newer seed-producing, turf-type, synthetic cultivar released in 2000 by OAES. Riviera was derived from the inter-pollinating of three parent plants, which were selected from a breeding population by C.M. Taliaferro in 1995. The parent plants were selected for visually assessed turf quality traits, cold hardiness, and seed production traits. Riviera was a top performer in the National Bermudagrass Tests of the NTEP from 1997 to 2006. In the 2002–2006 and 2007–2011 NTEP National Bermudagrass Tests, Riviera was selected as a seeded bermudagrass standard. Riviera has a unique combination of desirable traits including improved cold hardiness, high seed yield, and exceptional turf quality. Riviera turf plantings grown as far north as Warrensburg, Missouri, and Evansville, Indiana, have had minimal winter injuries, greened up early in spring, and exhibited high turf quality on golf courses, sports fields, and other turf areas (Taliaferro et al. 2004a).

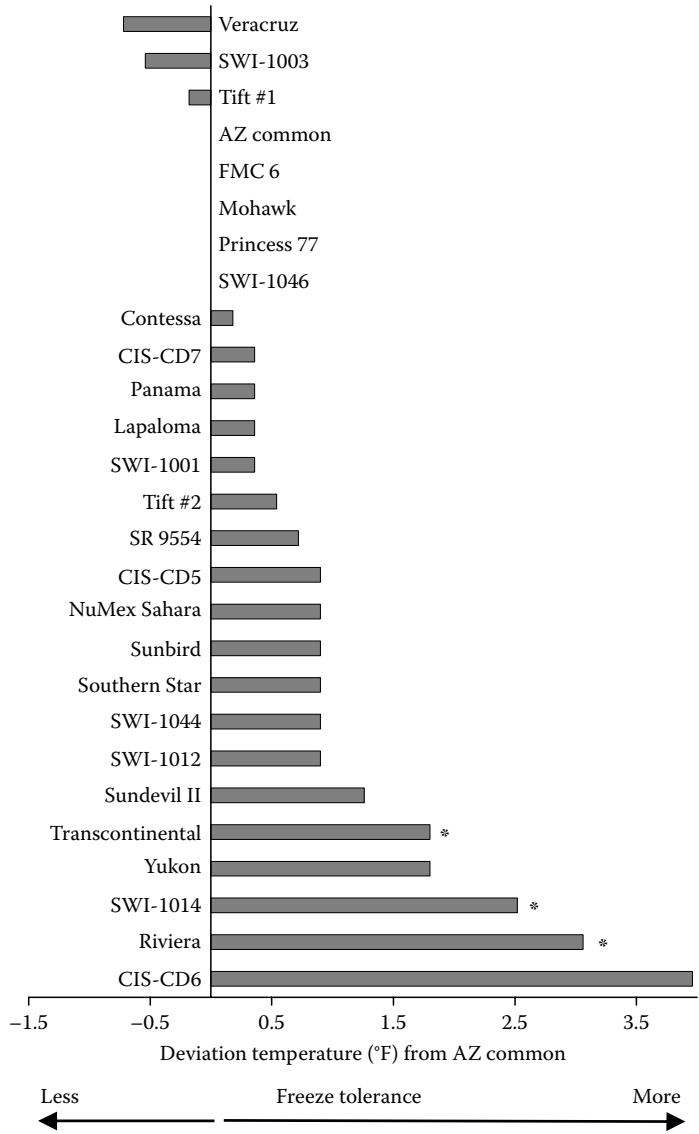
### 33.5 EVALUATION OF COLD HARDINESS: FIELD-BASED AND LABORATORY-BASED METHODS

Winter survival is a major concern when growing bermudagrass in the transition zone or regions of higher latitudes. Measuring the magnitude of freeze tolerance of released cultivars provides critical information for turf managers to select appropriate cultivars for specific regions, i.e., regional adaptation. Since quantitative evaluation of cold hardiness of bermudagrass germplasm is essential for breeders to develop cultivars with improved cold hardiness, technical approaches have been developed and employed for bermudagrass cold hardiness evaluation. Field-based visual estimates of winterkill based on percent ground cover of bermudagrass plots have been widely used in screening large progeny populations by breeders, in the NTEP (Morris 2009) and replicated studies (Martin et al. 2001, Wu et al. 2007, Stefaniak et al. 2009). In the field-based method, it is critical to evaluate the regrowth of overwintering bermudagrass stands at a proper time window, allowing all living buds to initiate growth but before the extension growth of new stolons from early growing plants. The majority of bermudagrass genotypes initiate active growth when soil temperatures exceed 10°C (Wu and Taliaferro 2009). The timing of field evaluation may vary from site to site, and from year to year at the same location.

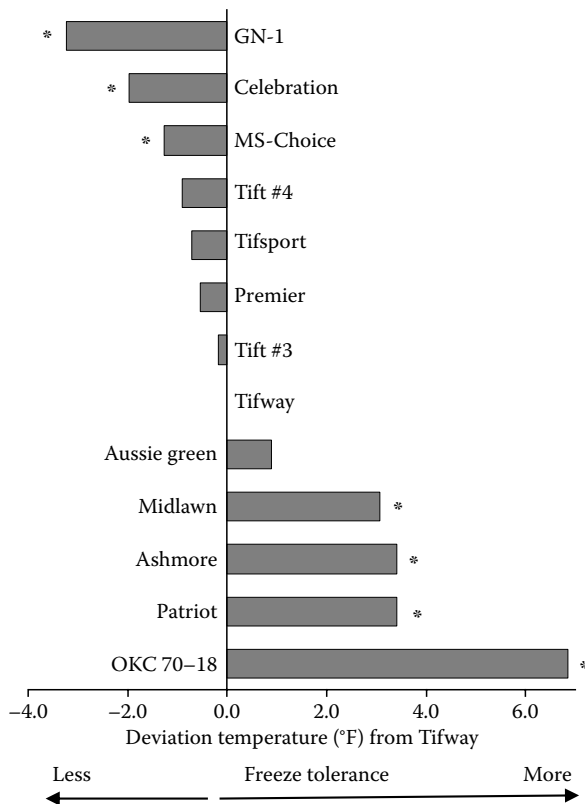
Visual estimates of plot ground cover in the spring following harsh winters, also known as test winters, is a direct measure of winter hardiness in bermudagrass. Observation following test winters provides an accurate index of survival, but does not distinguish between freeze tolerance, low-temperature avoidance, and resistance to secondary stresses. This classic approach has proven useful, especially for screening a large number of individuals of segregating populations. However, it may take several years to experience a test winter that distinguishes cold hardiness differences of bermudagrass plants (Anderson et al. 1988). Another concern with the field method is that weather conditions, especially during the acclimation period, vary and may have a marked influence on the acclimation state of plants to be tested. Exposure temperatures of plant tissues can vary tremendously, even with comparable air temperatures, if two locations differ in snow coverage and its insulating effect. Another factor that can make comparisons between field results difficult is the length of time that low temperatures persist because longer exposure durations significantly increase freeze damage to turf bermudagrasses (Anderson et al. 2003). Therefore, test winters are difficult to repeat over time and across locations (Anderson et al. 2005, 2007b).

As a repeatable alternative to field-based test winters, laboratory-based methods to estimate bermudagrass freeze tolerance have been developed. Ahring and Irving (1969) were among the first to establish a laboratory protocol to measure differences in bermudagrass cold hardiness, employing field-acclimated rhizomes, a laboratory-based freezing technique, and a viability test based on triphenyl tetrazolium chloride reduction. They reported relative cold hardiness of A-9957 from Yugoslavia and A-8153 from Afghanistan was higher than Midland and Greenfield, which were hardier than Coastal. Anderson et al. (1988) used electrolyte leakage and regrowth tests to estimate cold hardiness levels of field-acclimated Midiron and Tifgreen bermudagrass. They found Midiron to be hardier than Tifgreen. The authors preferred the regrowth test over the electrolyte leakage method although the latter procedure was rapid, required no greenhouse space, and the results of the two methods were in good agreement. The major reason was that the electrolyte leakage method involved intensive labor requirements to excise and wash crowns when a large number of cultivars are tested (Anderson et al. 1988). Viability testing based on regrowth was subsequently used for field-acclimated forage bermudagrass cultivars (Anderson and Taliaferro 1995). Anderson and Taliaferro (1995) reported that a majority of field cold acclimation occurred before November and deacclimation after active growth in May in Oklahoma. Their results indicated “Gordon’s Gift” bermudagrass was hardier than the other four cultivars Midland, Hardie, Tifton 44, and World Feeder with Tifton 44 being the least freeze tolerant. Recognizing that acclimation conditions in field plots are not reproducible and are subject to seasonal changes, Anderson et al. (1993) developed procedures for standardized cold acclimation of bermudagrass plants in controlled environment chambers, providing reproducibly acclimated plants for freezing and viability testing to evaluate subfreezing

temperature tolerance. The protocols have been refined and used successfully in quantifying freeze tolerance of new turf-type bermudagrass cultivars and breeding lines (Anderson and Taliaferro 2002, Anderson et al. 2002, 2003, 2007a). Relative freeze tolerance of many seeded and vegetatively propagated turf bermudagrasses is presented in Figures 33.1 and 33.2, respectively (adapted from Anderson et al. 2007a–c). Substantial variability in freeze tolerance exists in both forage and turf bermudagrass cultivars, indicating considerable progress made in breeding cold hardy bermudagrass cultivars for turf and forage purposes and indicating the availability to select harder cultivars for the transition zone. Cold tolerance of new seed-propagated cultivars, CIS-CD6,



**FIGURE 33.1** Freeze tolerance of seed-propagated bermudagrasses relative to “Arizona Common.” Deviation temperatures represent the  $T_{mid}$  value (midpoint of the survival-temperature response curve) of the cultivar minus the  $T_{mid}$  value for “Arizona Common.” Cultivars significantly different from “Arizona Common” are indicated by asterisk, based on mean comparisons using LSD at  $P \leq 0.05$ . (Adapted from Anderson, J.A. et al., *Appl. Turfgrass Sci.*, doi:10.1094/ATS-2007-0508-01-RS, 2007a; Anderson, J.A. et al., *GCSAA Golf Course Manage.*, 75(10), 110, 2007b; Anderson, J.A. et al., *USGA Turfgrass Environ. Res. Online* 6(18), 1, 2007c. With permission.)



**FIGURE 33.2** Freeze tolerance of vegetatively propagated bermudagrasses relative to “Tifway.” Deviation temperatures represent the  $T_{mid}$  value (midpoint of the survival-temperature response curve) of the cultivar minus the  $T_{mid}$  value for “Tifway.” Cultivars significantly different from “Tifway” are indicated by asterisk, based on mean comparisons using LSD at  $P \leq 0.05$ . (Adapted from Anderson, J.A. et al., *Appl. Turfgrass Sci.*, doi:10.1094/ATS-2007-0508-01-RS, 2007a; Anderson, J.A. et al., *GCSAA Golf Course Manage.*, 75(10), 110, 2007b; Anderson, J.A. et al., *USGA Turfgrass Environ. Res. Online* 6(18), 1, 2007c. With permission.)

Riviera, SWI-1014, Yukon and Transcontinental is significantly improved over Arizona Common. Numerous clonal turf cultivars, including the newer releases Patriot and Quickstand, have much better cold tolerance than the bench mark cultivar Tifway. Unfortunately, little progress has been made in the development of cold hardy putting-green type bermudagrass cultivars as Tifgreen continues to be the most hardy even though it was released half a century ago.

**33.6 INHERITANCE, PHYSIOLOGICAL, AND MOLECULAR MECHANISMS**

Although a number of cold hardy bermudagrass cultivars have been developed, inheritance of cold hardiness was poorly understood until recently. Using 114 Chinese bermudagrass accessions in a 2 year field experiment, we found estimates of genetic variance, genetic by year variance and environment variance components for winterkill were significantly different from zero, clearly indicating the trait is regulated by genetic factors, conditioned by environmental parameters, and modified by G by E interactions (Wu et al. 2007). Wu et al. (2007) reported highly significant negative correlation coefficients between winterkill and spring greenup, ranging from  $-0.52$  to  $-0.83$ . Stefaniak et al. (2009) reported the narrow-sense heritability estimates for spring greenup of combined 2 year data were 0.38, and from 0.75 to 0.91 with single year data, indicating a major portion of the genetic factors are additive in nature. Those results provide ample evidence for the potential to breed cold hardy cultivars using classical population improvement technology.



However, it is clear that tolerance of bermudagrass to freezing temperatures is not completely constitutively expressed. The greatest levels of cold hardiness in field-acclimated Midiron and Tifgreen were observed during December and January and the least cold hardiness was in early June among testing months from November in the first year to June the next year (Anderson et al. 1988). Davis and Gilbert (1970) reported Tifgreen and Tifdwarf bermudagrasses gradually increased in winter hardiness in the fall and early winter months. The two grasses sampled in field plots in January and February at Raleigh, North Carolina tolerated lower temperatures than during warmer months. Winter hardiness of the two grasses decreased when they started to grow in the spring. Cold acclimation during the fall involves physiological adjustments within the plant that maximize the ability of the plant to survive low-temperature stress (Beard 1998). Davis and Gilbert (1970) observed alterations in soluble proteins in Tifgreen and Tifdwarf bermudagrass during cold acclimation, although functions of the proteins were unknown. Gatschet et al. (1994) reported cold acclimation increased synthesis of several cold-regulated proteins in bermudagrass crowns. Cold-regulated proteins with molecular weight from 32 to 37kDa and from 20 to 26kDa were more abundant in Midiron than Tifgreen bermudagrass when cold acclimated, while proteins of about 34kDa decreased in crowns of both cultivars. One of the cold-regulated proteins with molecular weight 27kDa, designated as COR27, was subsequently identified as a chitinase according to its amino acid sequence homology with previously identified chitinase proteins (Gatschet et al. 1996). A chitinase gene that increased in expression during cold acclimation, *CynCHTI*, was cloned from Midiron bermudagrass by de los Reyes et al. (2001). The expression level of *CynCHTI* induced by cold acclimation was significantly higher in the freeze-tolerant bermudagrasses “MSU” and Midiron, compared with “Uganda” (*C. transvaalensis*), a less freeze-tolerant bermudagrass. Although the precise physiological roles of the chitinase and similar bermudagrass enzymes in freeze tolerance are still unknown, chitinases have exhibited antifreeze activity in other species (Moffatt et al. 2006). Samala et al. (1998) reported changes in polar membrane lipids of Midiron and U-3 bermudagrass during cold acclimation. Midiron had a quicker response and a nearly fourfold increase in the ratio of unsaturated fatty acids vs. saturated fatty acids compared with U-3 during cold acclimation, indicating some desaturases are responsive to cold acclimation and may be important in increasing cold hardiness in bermudagrass (Samala et al. 1998). Cold acclimation increased sugars and proline in both cold-tolerant “Riviera” and cold-susceptible “Princess-77,” while increase of total nonstructural carbohydrates (i.e., glucose, fructose, sucrose, and starch) and protein were observed only in Riviera, but not in Princess-77 during cold acclimation (Zhang et al. 2006). Zhang et al. (2006) believed rapid accumulation of carbon-rich and nitrogen-rich compounds during cold acclimation in bermudagrass appears to play an important role in increased freeze tolerance. Cold acclimation caused a substantial increase in abscisic acid (ABA) content and a 25 kDa dehydrin protein in leaves and stolons of bermudagrass and ABA levels of cold-tolerant bermudagrass were higher than those of relatively cold-intolerant bermudagrass (Zhang et al. 2008). Abscisic acid has been associated with cold acclimation for many decades, while dehydrins have recently been implicated in resistance to freezing and water deficit stresses (Patton et al. 2007). The linkage is not unexpected considering that water freezes extracellularly, causing a cellular level water stress during the freezing process. As knowledge of cold acclimation mechanisms accumulates, opportunities for targeted approaches for increasing freeze tolerance will accelerate.

### 33.7 FUTURE PROSPECTS IN BERMUDAGRASS COLD HARDINESS IMPROVEMENT

Bermudagrass is subject to winterkill when its use extends into temperate climatic regions. The remarkable success in the development of turf and forage bermudagrass cultivars enhanced in cold tolerance over the past 60 years has effectively lessened the risk of winterkill and increased bermudagrass use beyond its primary adaptation areas into the transition zone. Comparing the natural distribution of cold hardy bermudagrass germplasm with the current areas of use, it is not difficult to see the potential for development of new-generation cold hardy bermudagrass cultivars for turf

and forage use. Cold hardy bermudagrass germplasm are dispersed and persistent up to 45° latitude in the southern hemisphere and up to 45° to 53° latitudes in the northern hemisphere (Harlan and de Wet 1969), while geographic use of cold-tolerant bermudagrass cultivars is limited to about 38°N–39°N or lower latitudes (Taliaferro et al. 2004b). However, integration of improved cold hardiness and acceptable performance traits is a challenge. Bermudagrass germplasm with the best cold tolerance generally have less desirable performance characteristics for either turf or forage uses. Proven classical breeding techniques complemented with molecular tools (i.e., marker assisted selection) can be employed to integrate performance traits and cold hardiness into new cultivars.

Efforts should be focused on enhancing germplasm collection and deeper understanding of the mechanisms contributing to increased cold hardiness, and on combinational employment of classical breeding methods and biotechnology to develop new-generation cold hardy forage and turf cultivars in bermudagrass. Currently, in addition to the germplasm maintained in individual bermudagrass breeding programs, there are only 316 accessions of *C. dactylon*, 3 of *C. nlemfuensis*, and 18 of *C. transvaalensis* in the *Cynodon* collection of the USDA National Plant Germplasm System (NPGS 2009). Little is known about the cold hardiness in the collection. To exploit the maximum genetic diversity of the major species for breeding cold hardy turf and forage bermudagrass cultivars, more comprehensive collection of cold hardy *C. dactylon*, *C. nlemfuensis*, and *C. transvaalensis* is essential, especially those germplasm in original geographic regions. Remarkable success has been achieved in the precise evaluation of bermudagrass freeze tolerance and significant progress has been made in understanding the metabolic reactions during cold acclimation in bermudagrass. However, knowledge gaps still exist. For example, how many genes are involved in cold acclimation? Are there major genes responsible for cold hardiness, including transcription factors capable of activating arrays of stress responsive genes? The signal transduction pathways from recognition of environmental cues to production of stress-related proteins are largely unknown in bermudagrass. Integrative use of modern molecular tools, such as those developed in genomics, transcriptomics, proteomics, and metabolomics with current physiological and genetic efforts may shed new system-based lights on the mechanisms of cold hardiness in bermudagrass.

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# 34 Candidate Gene Expression Involved in Plant Drought Resistance

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## 34.1 INTRODUCTION

Drought stress triggers a wide range of responses in plants. This launches different kinds of signal transduction pathways that enable plants to survive stress conditions by adapting themselves at both the biochemical and physiological levels (Bray, 1993). During this process, a large number of genes with diverse functions are induced or suppressed (Kreps et al., 2002). Many of them have been transferred into plants and major agricultural crops that show the ability to promote drought tolerance (Zhang et al., 2004; Umezawa et al., 2006). Although a comprehensive understanding of gene regulation and functions under drought has not yet been achieved, identifying genes that play an important role in drought resistance will provide key insights into molecular adaptations of plants to drought stress.

## 34.2 GENES INVOLVED IN DROUGHT RESISTANCE

Shinozaki and Yamaguchi-Shinozaki (2007) classified the products of drought-inducible genes into two groups: regulatory proteins and functional proteins. In this chapter, the drought-induced candidate genes discussed have all been transferred to different plant species and have demonstrated their role in conferring drought tolerance.

### 34.2.1 GENES ENCODING REGULATORY PROTEINS

Loss of turgor pressure due to drought may activate some signal transduction pathways that further induce gene expression (Ding and Pickard, 1993). There are at least four independent

regulatory systems of gene expression in *Arabidopsis thaliana* under drought stress: two abscisic acid (ABA)-dependent systems and two ABA-independent systems (Shinozaki and Yamaguchi-Shinozaki, 2000).

#### 34.2.1.1 ABA-Dependent Systems

The activation of ABA biosynthesis is the first step needed for ABA-dependent regulatory networks. A key enzyme in the ABA biosynthesis pathway is 9-*cis*-epoxycarotenoid dioxygenase (NCED). Over-expression of *Arabidopsis NCED3* in transgenic *Arabidopsis thaliana* has improved drought tolerance by increasing the endogenous ABA level and enhancing the transcription of ABA-inducible or ABA-responsive genes (Iuchi et al., 2001). The aldehyde oxidase 3 encoded by *Arabidopsis AAO3* catalyzes the final step in ABA biosynthesis in leaves (Seo et al., 2000), and its expression is also up-regulated by drought stress (Zhu, 2002). The gene *ZEP* encoding zeaxanthin epoxidase and *MCSU* encoding molybdenum cofactor sulfuryase are involved in the ABA biosynthesis pathway. The up-regulated expressions of *ZEP* and *MCSU* under drought stress along with *NCED3* and *AAO3* have shown positive effects on stress tolerance (Xiong et al., 2002).

It is known that there are two ABA-dependent regulatory systems that activate the expression of many drought-inducible genes. One group involves transcription factors such as *MYC* and *MYB*. Cominelli et al. (2005) reported that *Arabidopsis MYB60* was specifically expressed in guard cells and negatively modulated during drought stress. A null mutation in *MYB60* reduces the constitutive stomatal opening and decreases wilting under water stress conditions. Compared to the wild type, the over-expression of rice (*Oryza sativa* L.) *MYB4* largely increases accumulation of several compatible solutes (glucose, fructose, sucrose, proline, glycine betaine (GB), and sinapoyl malate) (Mattana et al., 2005). The accumulation of these solutes could increase the capacity of osmotic adjustment that enhances drought tolerance. The other group of ABA-dependent regulatory systems involves the ABA-responsive element binding proteins (AREB) (also known as auxin-binding factors [ABFs]), which bind to ABA-responsive elements (ABRE) (Valliyodan and Nguyen, 2006). Plants over-expressing *ABF* in *Arabidopsis* show elevated expression levels of ABA/stress-regulated genes and become more sensitive to ABA and tolerant to dehydration (Kang et al., 2002). Among ABF family members, ABF2 is required for normal glucose response, whereas ABF3 and ABF4 play essential roles in ABA/stress responses (Kim et al., 2004).

ABA-dependent regulatory systems can also be mediated by one of the largest transcription factor NAC families. More than 100 members of this family have been identified in *Arabidopsis* and rice (Ooka et al., 2003; Fang et al., 2008). The expression of three members of the NAC transcription factor family (*NAC019*, *NAC055*, *NAC072*) are induced by drought, high salinity, and ABA in *Arabidopsis* (Tran et al., 2004). The over-expression of either one of the three transcription factors leads to up-regulation of several stress-inducible genes in the transgenic *Arabidopsis* plants with significantly enhanced drought tolerance. A rice *NAC045* is also induced by drought, high salt, low temperature, and ABA in leaves and roots, and over-expressing *NAC045* enhances drought tolerance (Zheng et al., 2009).

#### 34.2.1.2 ABA-Independent Systems

The ABA-independent regulatory system includes transcription factors such as *AP2/ERF* and *WRKY*, etc. The AP/ERF is a large gene family consisting of four subfamilies named AP2, CNF/DREB, ERF, and RAV (Sakamoto and Murata, 2002). Dehydration responsive element binding (DREB) protein plays an important role in plant resistance to dehydration stress. Over-expression of *DREB1* and *DREB2* improves drought tolerance in *Arabidopsis* (Liu et al., 1998). Over-expression of *DREB*-like genes also enhances drought tolerance in rice (Dubouzet et al., 2003) and in cotton (*Gossypium hirsutum*) (Gao et al., 2009). A new member of the *AP2/ERF* transcription factor family, the soybean *ERF3* gene, has been found to increase tolerance to drought stress in transgenic tobacco (*Nicotiana tabacum*) (Zhang et al., 2009). The *WRKY* genes encode a large group of transcription factors. Over 70 genes in *Arabidopsis* and over 100 gene members in rice have been found

in the *WRKY* family (Wu et al., 2005; Ramamoorthy et al., 2008). A recent study has shown that over-expression of *OsWRKY11* enhances heat and drought tolerance in transgenic rice seedlings (Wu et al., 2009).

The *Arabidopsis* *HARDY* gene (*HRD*) is a transcription factor of the AP2/EREBP family, discovered from a gain-of-function *Arabidopsis* mutant *hrd-D* (Karaba et al., 2007). The roots of this mutant exhibit enhanced strength, branching, and cortical cells, conferring drought resistance and salt tolerance. The expression of the *Arabidopsis* *HARDY* gene can improve water use efficiency in rice by increasing photosynthetic assimilation and reducing transpiration (Karaba et al., 2007).

All these ABA-dependent and -independent transcription factors are located relatively upstream along the signaling pathways. Since each factor may control more than one gene downstream, the genetic manipulation targeting these transcription factors is more likely to be successful in increasing drought tolerance.

### 34.2.2 GENES ENCODING FUNCTIONAL PROTEINS

Genes encoding the functional proteins associated with drought tolerance mainly consist of water channels and transporters, protection factors, detoxification enzymes, and key enzymes for osmolyte biosynthesis (Shinozaki and Yamaguchi-Shinozaki, 2007).

#### 34.2.2.1 Plasma Membrane Intrinsic Proteins

Plasma membrane intrinsic proteins (PIPs) are a type of aquaporin that facilitate water transport (Mahdiah et al., 2008). Most PIPs have a high level of expression under normal conditions, and their transcripts are usually down-regulated upon chronic drought stress in the leaves of *Arabidopsis* (Alexandersson et al., 2005). Mahdiah et al. (2008) proposed that tobacco *NtPIPI;1* and *NtPIP2;1* play an important role in water transport in roots. The expression of *NtPIPI;1* and *NtPIP2;1* has also been down-regulated to reduce osmotic hydraulic conductance in roots under drought stress. *AVPI* coding the vacuolar H<sup>+</sup>-pyrophosphatase (H<sup>+</sup>-PPase) is another example of a gene that mediates transport. Over-expression of *AVPI* in a commercial cultivar of tomato (*Solanum lycopersicum* L.) induces greater pyrophosphate-driven cation transport into root vacuolar fractions and enhances the development of a root system to resist and recover from stress (Park et al., 2005).

#### 34.2.2.2 Protection Factors

Members of the *LEA* (late-embryogenesis-abundant) family are expressed during desiccation phases of seed development (Baker et al., 1988) and can be induced in vegetative tissues during water or low-temperature stress (Bray, 1993). Most of them encode predominantly hydrophobic proteins (Zegzouti et al., 1997) and function in protecting other proteins and membranes, sequestering ions, and renaturing unfolded proteins (Bray, 1993). The *LEA* gene expression is induced by plant stress and ABA during seed maturation (Finkelstein et al., 2002) and vegetative tissues (Bies-Ethève et al., 2008). Their role in abiotic stress has been well documented for Groups 1, 2, and 3 *LEA* genes (Cheng et al., 2002; Yin et al., 2006; Xiao et al., 2007). Babu et al. (2004) found that the expression of barley (*Hordeum vulgare*) *HAV1*, an *LEA* gene, resulted in higher leaf relative water content and less reduction in transgenic rice plant growth under drought stress than non-transgenic (NT) plants. The transgenic line also showed relatively higher cell membrane protection than the NT line after 28 days of stress. A recent study by Dalala et al. (2009) demonstrated that transgenic *Arabidopsis* plants over-expressing *LEA4-1* from *Brassica napus* improved tolerance to salt and drought stress. Some proteins in the *LEA* gene family serve as dehydrins (Ismail et al., 1999). The expression of these dehydrins increases in plants under drought stress (Jiang and Huang, 2002; Watkinson et al., 2003). The up-regulated expression of *DHN-1* has also been observed in *Picea glauca* under drought stress or ABA treatment and in *Populus euramericana* under drought, salt, cold, and osmotic stress (Richard et al., 2000; Caruso et al., 2002), indicating its role in drought tolerance.

### 34.2.2.3 Antioxidant Enzymes

The reactive oxygen species (ROS) accumulates during drought, salt, and low-temperature stress, which could inactivate enzymes and damage proteins and membrane lipids, especially in mitochondria and chloroplast (Allen, 1995). Plants have evolved antioxidant defense systems to detoxify ROS. In enzymatic systems, superoxide dismutase (SOD) plays a central role in scavenging  $O_2^-$  to  $H_2O_2$  (Bowler et al., 1992). Peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) decompose  $H_2O_2$  to  $H_2O$  at different cellular locations (Asada, 1999; Mittler, 2002). There are three types of SOD isoenzymes based on their metal cofactors: copper/zinc (Cu/Zn), iron (Fe), and manganese (Mn), located in mitochondria and peroxisome, chloroplast and cytosolic, and chloroplast, respectively (Alscher et al., 2002).

The transformation of *MnSOD* from pea (*Pisum sativum* L.) into chloroplasts of rice reduces electrolyte leakage, suggesting that *MnSOD* plays an important role in improving drought tolerance by scavenging ROS in chloroplasts (Wang et al., 2005). Transgenic maize (*Zea Mays* L.) plants carrying *Arabidopsis FeSOD* show an enhanced oxidative stress tolerance (Van Breusegem et al., 1999). Over-expression of mitochondrial *MnSOD3.1* in *Brassica napus* exhibits higher total SOD activity along with an increased drought tolerance in the field and under artificial stress conditions (Gusta et al., 2009). Prashanth et al. (2008) found that the transgenic plants of rice over-expressing cDNA encoding a cytosolic Cu/ZnSOD from mangrove (*Avicennia marina*) were more tolerant to both drought and salinity. Genes encoding both Cu/ZnSOD and APX have been introduced into chloroplasts of potato (*Solanum tuberosum* L.), and transgenic plants have shown increased tolerance to heat, sulfur dioxide, and dehydration (Kwak et al., 2009). In addition, Li et al. (2009) reported that transgenic tobacco leaves over-expressed with a populus (*Populus* spp) peroxisomal APX (*PpAPX*) gene and had 80% higher APX activity under drought stress and significantly improved drought resistance at the vegetative stage. Expression of *GR* is strongly up-regulated in leaves under drought stress and recovery in a susceptible cultivar but remains stable in the tolerant cultivar of cowpea (*Vigna sinensis*) leaves (Torres-Franlin et al., 2008). All these results demonstrate that enhanced antioxidant enzyme defense systems confer drought tolerance by minimizing oxidative injury potentially caused by ROS under stress conditions.

### 34.2.2.4 Osmolyte Biosynthesis

Osmolytes are a group of small molecules (e.g., proline, sugars, organic acids, ions, and GB) that can stabilize proteins and membranes by preventing them from denaturing (Yancey et al., 1982). They are also involved in osmotic adjustment, which contributes to water retention in the plant cells and increases water-use efficiency and crop yield under drought stress. In some cases, drought-susceptible plant species or cultivars fail to survive drought because they are unable to synthesize these osmolytes (Valliyodan and Nguyen, 2006).

Proline is highly accumulated in some stress-tolerant species under drought stress (Türkan et al., 2005). The glutamate pathway of proline biosynthesis is predominant under osmotic stresses (Delauney and Verma, 1993).  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS) and  $\Delta^1$ -pyrroline-5-carboxylate reductase (P5CR) are two important enzymes involved in the proline biosynthesis pathway. Petunias (*Petunia hybrida* cv. Mitchell) transferred with *Arabidopsis AtP5CS* and rice *OsP5CS* show a much higher proline concentration, which allows the plants to tolerate a longer period of drought stress (Yamada et al., 2005). Transgenic soybean (*Glycine max* cv. Ibis) plants carrying *P5CR* show high leaf relative water content and proline concentration and are more drought tolerant (De Ronde et al., 2004). Bhatnagar-Mathur et al. (2009) also found that transgenic chickpea (*Cicer arietinum* L.) with the *P5CSF129A* gene increased two- to six-fold the proline concentration and extracted more water than their untransformed parents.

GB is a quaternary ammonium compound and an important compatible solute that accumulates and protects plants against abiotic stresses (Khan et al., 2009). Exogenous applications of GB



increase drought tolerance in a number of crop species (Mäkelä et al., 1996; Xing and Rajashekar, 1999; Ashraf and Foolad, 2007). There are three pathways involved in GB biosynthesis: (1) the choline monooxygenase/betaine aldehyde dehydrogenase pathway (CMO/BADH), (2) the choline dehydrogenase/betaine aldehyde dehydrogenase pathway (CDH/BADH), and (3) the direct choline oxidase pathway (COD) (Cherian et al., 2006). Over-expression of CDH in maize and cotton improves drought tolerance (Quan et al., 2004; Lv et al., 2007). An increased tolerance to a toxic level of choline and to salt/drought stress has also been observed by introducing a gene encoding CMO from beet (*Beta vulgaris* L.) into tobacco (cv. Wisconsin 38) via plastid transformation (Zhang et al., 2008). Si et al. (2007) constructed plasmid with the gene encoding BADH and successfully obtained transgenic tobacco plants with better drought and salt resistance. Crops such as rice, potato, and tomato are unable to accumulate GB, so the genetic engineering of GB biosynthesis for these economically important species is necessary.

The accumulation of compounds related to sugar metabolism also plays an important role in osmotic adjustment and drought tolerance. The most extensively studied sugar-related compounds consist of sugar alcohol mannitol, trehalose, fructans, *D*-ononitol, and *D*-pinitol (Cherian et al., 2006). The gene *mtlD* encodes mannitol-1-phosphate dehydrogenase, catalyzing the reversible conversion of fru-6-phosphate to mannitol-1-phosphate. Then mannitol-1-phosphate can be converted to mannitol via nonspecific phosphatases in transgenic plants (Thomas et al., 1995). Abebe et al. (2003) reported that the over-expression of *mtlD* improved wheat growth by influencing the fresh weight, dry weight, plant height, and flag leaf length. The mechanism of its protection from stress could also be due to scavenging of hydroxyl radicals (OH<sup>-</sup>) or the stabilization of macromolecules (Shen et al., 1997).

Trehalose is an osmoprotectant that maintains lipids in a fluid phase and stabilizes structural and functional proteins under water-deficit conditions (Crowe et al., 1992; Elbein et al., 2003). It has been known that trehalose biosynthesis is involved in trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) (Avonce et al., 2006). The over-expression of genes encoding trehalose biosynthetic enzymes improves the drought tolerance of tobacco and potato (Holmström et al., 1996; Yeo et al., 2000). The expression of yeast TPS1-TPS2 in *Arabidopsis* confers tolerance on several abiotic stresses (Miranda et al., 2007). A significant increased drought tolerance has been observed in alfalfa (*Medicago sativa* L.) in the over-expression of *TPS1-TPS2* (Suárez et al., 2009).

Fructans are soluble polymers of fructose. The synthesis of these structurally complex polymers is achieved via the cooperation of a suite of fructosyltransferases (FTs) (Valluru and Van den Ende, 2008). Synthesized from sucrose by FTs, they are recognized as protective agents against abiotic stresses, including cold and drought (Valluru and Van den Ende, 2008). *SacB* gene from *Bacillus subtilis* is involved in fructan biosynthesis. Pilon-Smits et al. (1999) reported that the over-expression of *SacB* in sugar beet showed a higher dry weight under drought stress than untransformed beets. A putative sucrose:fructan 6-fructosyltransferase (6-SFT) gene from a Patagonian grass species, *Bromus pictus*, is associated with drought and cold temperatures (Del Viso et al., 2009). The transgenic perennial ryegrass (*Lolium perenne* L.) and rice over-expressing wheat (*Triticum aestivum* L.) fructosyltransferase genes, *6-SFT* and *1-SST*, increase freezing and chilling tolerance (Hisano et al., 2004; Kawakami et al. 2008).

#### 34.2.2.5 Other Functions

Genes encoding functional proteins also include proteins involved in wax/cuticle biosynthesis. The cuticle is a lipid barrier, protecting vascular plants and some bryophytes against environmental stresses such as drought and pathogen attack (Rowland et al., 2007). Although the mechanism of its synthesis, assembly, and deposition in epidermal cells is not well understood, studies on mutants in maize and *Arabidopsis* have identified many genes involved in wax production, including fatty acid elongase, integral membrane enzymes, regulatory proteins, acyltransferases, and transporters.

*Arabidopsis* *CER3* is an important gene for cuticular wax biosynthesis and is allelic to *WAX2/YRE/FLPI* (Rowland et al., 2007). Chen et al. (2003) predicted that *WAX2* had a metabolic function

associated with both cuticle membrane and wax synthesis. Over-expression of *WXPI* and *WXP2* in *Arabidopsis* significantly increased cuticular wax deposition on leaves of 4- and 6-week-old transgenic plants with enhanced whole plant tolerance to drought (Zhang et al., 2007). They further concluded that only *WXPI* was a useful candidate gene for improving plant drought because *WXP1* did not affect chlorophyll leaching, plant growth, or development.

*ERECTA* is a putative leucine-rich repeat receptor-like kinase and has been identified in *Arabidopsis*, which can regulate transpiration by increasing stomatal density and altering leaf epidermal cell expansion, mesophyll cell proliferation, and cell–cell contact (Masle et al., 2005). Transferring of *ERECTA* may improve drought tolerance by regulating transpiration efficiency of crop plants. This deserves further investigation.

### 34.3 SUMMARY

The candidate genes involved in drought resistance encode regulatory and functional proteins from a diverse family. Transformation of these genes has been shown to enhance drought tolerance in different plant species. Improvement of drought-tolerant plant materials can be challenging due to the complex nature of drought stress in the field, the large genotype by environmental interaction, the complex genetic background of some species, and the lack of understanding of key candidate genes controlling drought tolerance. However, genes identified in the model plant species could provide guidance in selecting candidate genes and investigating gene expression and function in crop species. Ultimately, genes and markers linked to drought tolerance can be identified through modern techniques such as association mapping and can be applied to genetic improvement of drought tolerance for agricultural crops.

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## *Part VII*

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*Examples of Empirical Investigations  
of Specific Plants and Crops Grown  
in Salt, Drought, and Other  
Environmental Stress Conditions*

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# 35 Responses of Green Beans (*Phaseolus vulgaris* L.) in Terms of Dry Matter Production, Nitrogen Uptake, and Water Absorption under Salt-Stress Conditions

Mohammad Pessarakli

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## 35.1 INTRODUCTION

The gradual progress of desertification due to detrimental effects of natural stress factors such as low precipitation, long-term drought, heat, and erosion coupled with improper human activities as a result of overgrazing, overutilization of land and the application of insufficient management decisions and improper agricultural practices, urbanization, and industrial activities has left extensive arable lands at potential risk of conversion to unusable soils. These problems are more severe in arid and semiarid regions whose soils already encounter salinity and sodicity problems, and are more vulnerable to stress conditions.

Salinity stress is one of the major abiotic stresses limiting crop productivity and the geographical distribution of many important plants/crops worldwide. Accumulation of high soluble salts in a soil can significantly decrease the value and productivity of agricultural lands. Salt and water stress have been recognized as major agricultural problems, especially in arid and semiarid regions since a long time ago. Retardation of crop yield by salinization has also been known for a long time. Since the early 1900s, various investigations of effects of salts on plant/crop growth have been



undertaken, covering a range of aspects from plant response to salinity to salt behavior in soils [2,3, 5–7,14,15,20,25,27,31,36–38,42,44,52,53,60,65,67,69,77,85,86,89,92,93,96,97,101,112,118,121,122, 125,126,131,133,134,152,156,157,164,167,168,171,172,175–179,184,185,189]. Physiological studies have revealed that the major effects of salinity on plant growth retardation are osmotic and specific ion effects [4,17,29,33,34,45–47,50,55,56,68,81,88,94,95,99,101,112,115,116,146,148,149,159,173]. Furthermore, reduced nutrient uptake by plants grown in saline environments has been observed in several species of plants [9,10,20,26,29,37,44,47,53,61,62,65,70,78,81,87,88,99,112,122,128–130, 133,135–138,144,147,159,166]. Differences in salt tolerance among plant species also have been long recognized [17,21,23,28,48,68,72–74,76,81,82,84,90,91,95,98,103–106,109,114,116,117,148, 150,160,165,181,182,186]. Although the scientists in agriculture have started to work on salinity tolerance of plants over 50 years ago, since early 1950s, there is still a great deal of interest in working on this subject by the researchers. For some sample reports on this subject in the last two decades, see Refs. [3–20,22,25,26,29–31,38–44,49–52,54,57–59,63,65,69–71,77–79,83,85,86,89–93,96,97,100–102, 107,108,111–113,117,119,120,122–127,131–134,139–145,149,151–155,157,158,161–164,166–185, 187–191]. According to Qadir et al. [141], cultivation of salt-tolerant grasses in a saline or saline–sodic soil may mobilize the native lime ( $\text{CaCO}_3$ ) in these soils through root action. This may substitute the chemical approach for reclamation of such soils. Apte and Thomas [12] reported that simultaneous application of halotolerant nitrogen-fixing cyanobacteria during crop growth seems to be an attractive possibility for reclamation and improvement of saline soils, especially since it can also supplement the nitrogen requirement of the crop. De Villiers et al. [51], also assessing salinity tolerance of different plant species, found that the perennials seemed to be better suited for rehabilitation purposes under saline soil conditions. However, the role that salt tolerance plays in causing differences in growth and development, nutrient uptake, and metabolism between various plants, among plant species, and at different stages of growth is still a major concern among investigators, and has not been fully understood. Discovery of the physiological basis of salt tolerance in crops and the use of this knowledge to obtain more tolerant cultivars by modern plant breeding procedures should result in substantial increases in world food production.

The effect of salt stress on nutrient elements utilization and nutrition as well as metabolism in plants has been studied for various plants using different methods. The results are still inconclusive. However, the change in nutrient metabolism induced by excess salt is commonly accepted among scientists as one of the most important factors responsible for abnormal plant metabolism and reduced growth. Bernstein et al. [35] found that despite the decrease in total N uptake, leaf N concentration of some grain and vegetable crops increased with increasing salinity at all N fertilization levels. Increase in the N concentration of corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) plants under salinity stress was reported by Khalil et al. [87]. The uptake and metabolism of  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  in red kidney beans (*Phaseolus vulgaris* L.) was adversely affected by both salt and water stress at  $-0.4$  MPa osmotic potential [61,62,147]. Reduced  $^{15}\text{N}$  uptake and metabolism as well as impaired protein synthesis under stress conditions, by various crops, have also been reported by several other investigators; Helal and Mengel [75] (barley, *Hordeum vulgare* L.); Pessaraki and Tucker [137] and Al-Rawahy et al. [9,10] (tomato, *Lycopersicon esculentum* Mill.); Pessaraki and Tucker [138] (eggplant, *Solanum melongena* L.); Pessaraki et al. [130] (corn); Pessaraki [122] and Pessaraki et al. [128,129] (green beans, *Phaseolus vulgaris* L.). However, Pessaraki and Tucker [135,136] found that  $^{15}\text{N}$  uptake and protein synthesis by cotton plants increased under low levels ( $-0.4$  MPa osmotic potential) of NaCl salinity. Increased total N concentration of plants grown in saline substrate also was reported by Bernstein and Pearson [36].

To explain these different results, a dilution or concentration effect (depending on the relative severity of salt stress on growth or nutrient, i.e., N uptake) was reported as a cause of the fluctuations in N content or concentration in plants [61,122,135,137,138].

Among the various environmental stress factors, salinity appears to have been given more attention than any other factors both in the past and in the present time. This is clearly seen from the continuous investigations and the voluminous reports that are continuously being generated on this subject.

Hundreds of publications are annually added to the literature on this subject, dealing with plant and crop stress caused by salinity. For some recent reports during the last decades, see Refs. [3–7,11,13–15,17–20,22,25,26,30,31,38–44,49,50,52,54,57–59,63,65,69,70,77–79,83,85,86,89,90,92,93,96,97,100–102,107,108,111–113,117,119,120,123–126,131–134,142–145,151–155,157,161–164,166–168,170,172–174,176,178,179,183–185,187–191].

Despite the voluminous publications dealing with the effects of salt stress on plant growth and nutrient (i.e., N) nutrition, the literature concerning this issue on green beans is scarce. The reports of Gauch and Wadleigh [64], Balasubramanian and Sinha [27], Bhivare and Nimbalkar [37], Csizinsky [47], Coons and Pratt [45], Wignarajah [177], Frota and Tucker [61,62], Saad [147], Hoffman et al. [76], Maliwal and Paliwal [104], Harbir-Singh et al. [73], Salim and Pitman [150], Ashraf and Rasul [21], Alislail and Bartels [8], Velagaleti et al. [169], Wang and Shannon [175], van Hoorn et al. [167], Lopez et al. [97], Papiernik et al. [119], Savvas et al. [154], Bahmaniar and Sepanlou [26], and Kant et al. [83] deal primarily with the effects of salinity on the growth and/or chemical composition of other types of beans and are not concerned with green beans. Among the cited references in this chapter, in addition to the author's own research work, only that of Bernstein and Pearson [36] reported the influence of exchangeable sodium ions on the yield and chemical composition of green beans. However, Bernstein and Pearson's report [36] was not concerned with N (labeled or nonlabeled) uptake and metabolism by the reported plant species. Thus, green beans were selected to be covered in this chapter primarily because they are classified as salt-sensitive plants [24]. Also, the effects of salinity on the growth and nutrient uptake and utilization by these plants have not been studied and documented sufficiently. In addition, these salt-sensitive plant species were selected for this chapter to compile information regarding responses of different plant species, which are discussed by different authors in this book. This information is being compiled in a volume to assist the readers in comparing all these various salt-tolerant plant types under the stressful environmental conditions.

Thus, this chapter is concerned with growth, nitrogen (total and  $^{15}\text{N}$ ) uptake, protein synthesis, and water absorption by three cultivars (Tender Improved, Slim Green, and Kentucky Wonder) of green beans at the vegetative stage of growth under normal and NaCl-stress conditions with the following objectives:

1. To compare the growth of these cultivars by evaluating their dry-matter yield under normal and NaCl-stress conditions.
2. To compare total N and  $^{15}\text{N}$  uptake and distribution in plant roots and shoots by these cultivars as affected by salinity.
3. To evaluate protein synthesis by these plant species under normal and salt-stress conditions.
4. To study the water absorption by these cultivars as influenced by sodium chloride (NaCl) stress.

## 35.2 FACTORS EVALUATED REGARDING THE RESPONSES OF GREEN BEANS TO SALT STRESS

### 35.2.1 DRY-MATTER PRODUCTION

The effects of NaCl salinity on dry-matter production of the three cultivars of green beans have been examined in several studies [122,128,129,140]. All these studies reported that the NaCl stress significantly reduced total dry-matter yield for all three cultivars, but Tender Improved was the least severely affected at all salinity levels (Table 35.1, data from Ref. [128]). The degree of reduction in dry-matter yield increased with increasing salt-stress level and over time. Other investigators have also reported reductions in dry matter production and decrease in yields of other bean cultivars [8,21,36,37,45,47,61,62,64,73,83,104,119,147,150] and a number of other crops [2,3,9,10,14,31,35,42,52,60,66,69, 85,118,121,130,135–139,167,168,171–173,176].

**TABLE 35.1**  
**Dry Matter Yield of Three Green Bean Cultivars under Various NaCl Stress Levels at Different Harvest Times**

| Cultivar        | Salt Stress (Osmotic Potential) (MPa) | Dry Weight of Plant Parts <sup>a</sup> (g) |      |      |       |      |      |
|-----------------|---------------------------------------|--------------------------------------------|------|------|-------|------|------|
|                 |                                       | Harvest <sup>b</sup>                       |      |      |       |      |      |
|                 |                                       | Shoots                                     |      |      | Roots |      |      |
|                 |                                       | 1                                          | 2    | 3    | 1     | 2    | 3    |
| Tender Improved | Control (−0.03)                       | 3.12                                       | 5.18 | 7.25 | 0.84  | 0.98 | 1.48 |
|                 | −0.25                                 | 2.73                                       | 4.45 | 6.53 | 0.68  | 0.93 | 1.45 |
|                 | −0.50                                 | 1.92                                       | 3.64 | 4.56 | 0.46  | 0.81 | 1.14 |
| Slim Green      | Control (−0.03)                       | 1.76                                       | 4.24 | 7.22 | 0.36  | 0.78 | 1.51 |
|                 | −0.25                                 | 1.32                                       | 2.16 | 3.51 | 0.34  | 0.49 | 0.88 |
|                 | −0.50                                 | 0.76                                       | 0.92 | 1.34 | 0.21  | 0.32 | 0.41 |
| Kentucky Wonder | Control (−0.03)                       | 3.12                                       | 4.33 | 7.54 | 0.67  | 0.85 | 1.53 |
|                 | −0.25                                 | 1.67                                       | 2.45 | 3.18 | 0.55  | 0.77 | 1.28 |
|                 | −0.50                                 | 0.95                                       | 1.24 | 2.41 | 0.43  | 0.51 | 0.72 |
| LSD (0.05)*     |                                       | 0.42                                       | 0.76 | 0.96 | 0.18  | 0.24 | 0.35 |

Source: Pessarakli, M. et al., *J. Plant Nutr.*, 12(10), 1105, 1989a.  
<sup>a</sup> Represents the means for pots containing 2 plants with 3 replications.  
<sup>b</sup> Harvests 1, 2, and 3 are for 5, 10, and 15 days <sup>15</sup>N uptake periods, respectively.  
\* Represents the least significant difference between the treatment means at the 0.05 level of confidence.

Under NaCl stress, shoot and root growth were substantially lower for the Slim Green and Kentucky Wonder cultivars as compared with the Tender Improved [122,128,129,140]. This phenomenon indicates the presence of significant interaction effects between salinity and cultivars. Roots appear to be affected less than shoots by salt stress for all cultivars.

Dry-matter production and growth period were linearly correlated (*r*<sup>2</sup> values ranged from 0.89 to 0.99 for different treatments) [122]. Several studies conducted on these cultivars of green beans [122,128,129,140] reported that for all cultivars, dry-matter yield increased as growth period progressed.

**35.2.2 TOTAL N UPTAKE BY PLANTS**

According to Pessarakli [122] and Pessarakli et al. [128,129], total N uptake by green bean plants was significantly decreased with increasing salinity of the nutrient solutions for all cultivars, at all three harvests. The results of Pessarakli’s study [122] are presented here (Table 35.2). Slim Green contained substantially lower total N than the other two cultivars at each harvest for all corresponding treatments, except for the control shoots at the third harvest. The uptake values were markedly lower at the first harvest for this cultivar, indicating slower initial N uptake and slower early growth rate for the Slim Green cultivar. Several nitrogen uptake studies conducted on these cultivars of green beans [122,128,129] reported that for all cultivars, shoots contained substantially more total N than roots, probably due to the larger dry weights of shoots than roots (larger sink size).

Reduction in total N uptake was similar to the reduction pattern for total dry-matter yield by plants under NaCl stress. The similar reduction pattern for total N uptake and dry-matter yield indicates that the major portion of the absorbed N was incorporated into protein and contributed to plant growth and development. As N uptake decreased, dry-matter yield also decreased under

**TABLE 35.2**  
**Total N Uptake of Plant Parts of Three Green Bean Cultivars as Affected by Three Levels of NaCl Stress at Three Harvest Times**

|                                |                                       | Total N Content of Plant Parts (mg N Pot <sup>-1</sup> ) <sup>a</sup> |       |       |       |      |      |
|--------------------------------|---------------------------------------|-----------------------------------------------------------------------|-------|-------|-------|------|------|
| Cultivar                       | Salt Stress (Osmotic Potential) (MPa) | Harvest <sup>b</sup>                                                  |       |       |       |      |      |
|                                |                                       | Shoots                                                                |       |       | Roots |      |      |
|                                |                                       | 1                                                                     | 2     | 3     | 1     | 2    | 3    |
| Tender Improved                | Control (−0.03)                       | 104.8                                                                 | 146.2 | 210.4 | 24.4  | 27.6 | 40.6 |
|                                | −0.25                                 | 68.6                                                                  | 97.4  | 184.2 | 18.8  | 26.8 | 38.9 |
|                                | −0.50                                 | 42.8                                                                  | 82.8  | 113.4 | 13.1  | 24.1 | 28.8 |
| Slim Green                     | Control (−0.03)                       | 46.6                                                                  | 118.8 | 215.3 | 10.4  | 23.5 | 44.5 |
|                                | −0.25                                 | 33.2                                                                  | 58.7  | 89.2  | 9.3   | 14.4 | 22.6 |
|                                | −0.50                                 | 18.2                                                                  | 24.5  | 35.7  | 6.0   | 8.7  | 10.2 |
| Kentucky Wonder                | Control (−0.03)                       | 99.2                                                                  | 126.2 | 204.5 | 21.6  | 27.1 | 48.3 |
|                                | −0.25                                 | 48.6                                                                  | 71.1  | 86.4  | 16.8  | 21.1 | 38.1 |
|                                | −0.50                                 | 26.6                                                                  | 32.6  | 65.6  | 12.5  | 14.7 | 20.4 |
| LSD (0.05) salinity × cultivar |                                       | 3.4                                                                   | 5.3   | 13.1  | 1.6   | 2.3  | 3.1  |

*Summary of the significance of variance sources*

|              |    |    |    |    |    |    |
|--------------|----|----|----|----|----|----|
| Cultivar (C) | ** | *  | *  | ** | *  | *  |
| Salinity (S) | ** | ** | ** | ** | ** | ** |
| C × S        | ** | ** | ** | ** | ** | ** |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.

<sup>a</sup> Represents the means for pots containing two plants with three replicates.

<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.

\*, \*\* Significant at *P* = 0.05 and 0.01, respectively.

the NaCl-stress condition. This is supported by reports of several investigators [61,62,64,67,75, 127–129,133,135,136,147], which indicated that changes in N metabolism caused by salinity stress is one of the most important factors responsible for abnormal plant metabolism, reduced growth, and decreased crop yield.

**35.2.3 TOTAL N CONCENTRATION IN PLANT TISSUES**

All three studies conducted by Pessarakli [122] and Pessarakli et al. [128,129] reported that total N concentrations in all three cultivars generally were lower in plants subjected to salinity, especially at the highest NaCl stress levels, as compared with controls. Table 35.3, obtained from Ref. [122], indicates this finding. However, for a salt-tolerant cotton plant, N concentration was significantly higher in NaCl-stressed plants, even at a higher level of salinity (−0.8 MPa osmotic potential), as observed by Pessarakli and Tucker [135,136]. Increase in N concentration of corn and cotton plants under salt-stress conditions was also reported by Khalil et al. [87]. Therefore, differences in N concentrations of these different crops (cotton as compared with green beans) under salt stress are probably due to differences in their salt tolerance. At each stress level, total N concentration of Tender Improved generally tended to be lower than those of the other cultivars [122,128,129]. This is probably due to a dilution effect, since Tender Improved produced significantly higher dry matter than the other cultivars at each stress level for each harvest. Total N concentration of roots was generally higher than that of the shoots at each harvest, for each cultivar, for any corresponding treatment, except for the control Tender Improved plants [122].

**TABLE 35.3**  
**Nitrogen Concentration of Plant Parts of Three Green Bean Cultivars**  
**as Affected by Three Levels of NaCl Stress at Three Harvest Times**

| Cultivar                                               | Salt Stress (Osmotic Potential) (MPa) | Nitrogen Concentration of Plant Parts<br>(mg N g <sup>-1</sup> Dry Weight) <sup>a</sup> |      |      |       |      |      |
|--------------------------------------------------------|---------------------------------------|-----------------------------------------------------------------------------------------|------|------|-------|------|------|
|                                                        |                                       | Harvest <sup>b</sup>                                                                    |      |      |       |      |      |
|                                                        |                                       | Shoots                                                                                  |      |      | Roots |      |      |
|                                                        |                                       | 1                                                                                       | 2    | 3    | 1     | 2    | 3    |
| Tender Improved                                        | Control (−0.03)                       | 33.6                                                                                    | 28.2 | 29.0 | 29.0  | 28.2 | 27.4 |
|                                                        | −0.25                                 | 25.1                                                                                    | 21.9 | 28.2 | 27.6  | 28.8 | 26.8 |
|                                                        | −0.50                                 | 22.3                                                                                    | 22.7 | 24.9 | 28.4  | 29.7 | 25.3 |
| Slim Green                                             | Control (−0.03)                       | 26.5                                                                                    | 28.0 | 29.8 | 28.9  | 30.1 | 29.5 |
|                                                        | −0.25                                 | 25.2                                                                                    | 27.2 | 25.4 | 27.4  | 29.4 | 25.7 |
|                                                        | −0.50                                 | 23.9                                                                                    | 26.6 | 26.6 | 28.6  | 27.2 | 24.9 |
| Kentucky Wonder                                        | Control (−0.03)                       | 31.8                                                                                    | 29.1 | 27.1 | 32.2  | 31.9 | 31.6 |
|                                                        | −0.25                                 | 29.1                                                                                    | 29.0 | 27.2 | 30.5  | 27.4 | 29.8 |
|                                                        | −0.50                                 | 28.0                                                                                    | 26.3 | 27.2 | 29.1  | 28.8 | 28.3 |
| LSD (0.05) salinity × cultivar                         |                                       | 1.4                                                                                     | 1.5  | 1.3  | 1.3   | 1.3  | 1.3  |
| <i>Summary of the significance of variance sources</i> |                                       |                                                                                         |      |      |       |      |      |
| Cultivar (C)                                           |                                       | **                                                                                      | NS   | *    | *     | *    | **   |
| Salinity (S)                                           |                                       | *                                                                                       | *    | *    | *     | *    | **   |
| C × S                                                  |                                       | *                                                                                       | *    | *    | *     | *    | **   |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.

<sup>a</sup> Represents the means for pots containing two plants with three replicates.

<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.

\*, \*\* Significant at *P* = 0.05 and 0.01, respectively.

### 35.2.4 NITROGEN-15 UPTAKE BY PLANTS, AND DISTRIBUTION OF <sup>15</sup>N IN SHOOTS AND ROOTS

The results of several studies [122,128,129] on different cultivars of green beans showed that total-<sup>15</sup>N uptake by plants was decreased with increasing salinity of nutrient solutions at all three harvests, for all three cultivars. The <sup>15</sup>N results of an experiment completed by Pessarakli [122] are presented here in Table 35.4. Reduction in <sup>15</sup>N uptake followed the same reduction patterns as total N and dry-matter yield, under stress conditions. This is an indication that the absorbed <sup>15</sup>N was incorporated into protein and contributed to plant growth and development, as reflected in dry-matter production. The Slim Green cultivar absorbed the least amount of <sup>15</sup>N under NaCl stress conditions. The absorbed <sup>15</sup>N values were higher for Kentucky Wonder and generally highest for Tender Improved, under stress conditions. However, Tender Improved contained significantly lower <sup>15</sup>N in both shoots and roots, at the third harvest, under normal (nonsaline) condition as compared with the other two cultivars [122]. Substantial differences between the <sup>15</sup>N uptakes by the cultivars at each salinity level (Table 35.4) imply a significant interaction effect between salinity and cultivars at each harvest, for each plant part. Significant decreases in <sup>15</sup>N uptake by these plants, under high salinity level, is in agreement with experimental data obtained with red kidney beans [61,147], cotton [135], barley [75], tomato [9,10,137], and eggplant [138]. However, low level of NaCl salinity (−0.4 MPa osmotic potential), in cotton, slightly enhanced <sup>15</sup>N uptake [135]. Similar results were reported for saltgrass (*Distichlis spicata*), a true halophyte by Pessarakli et al. [133]. This phenomenon is probably due to the difference in the salt tolerance of these different plant types (cotton as compared with green beans).

**TABLE 35.4**  
**Nitrogen (<sup>15</sup>N) Content of Plant Parts and Shoot to Root <sup>15</sup>N Ratios of Three Green Bean Cultivars as Affected by Three Levels of NaCl Stress at Three Harvest Times**

| Cultivar                       | Salt Stress (Osmotic Potential) (MPa) | <sup>15</sup> N Content of Plant Parts (mg <sup>15</sup> N Pot <sup>-1</sup> ) <sup>a</sup> |      |      |       |      |      |                                     |      |      |
|--------------------------------|---------------------------------------|---------------------------------------------------------------------------------------------|------|------|-------|------|------|-------------------------------------|------|------|
|                                |                                       | Harvest <sup>b</sup>                                                                        |      |      |       |      |      | Shoot to Root <sup>15</sup> N Ratio |      |      |
|                                |                                       | Shoots                                                                                      |      |      | Roots |      |      | 1                                   | 2    | 3    |
|                                |                                       | 1                                                                                           | 2    | 3    | 1     | 2    | 3    |                                     |      |      |
| Tender Improved                | Control (−0.03)                       | 3.23                                                                                        | 5.58 | 8.57 | 0.98  | 1.19 | 1.91 | 3.30                                | 4.69 | 4.49 |
|                                | −0.25                                 | 1.56                                                                                        | 3.37 | 7.22 | 0.69  | 1.24 | 1.88 | 2.12                                | 2.72 | 3.84 |
|                                | −0.50                                 | 1.08                                                                                        | 2.94 | 4.15 | 0.38  | 1.06 | 1.30 | 2.84                                | 2.77 | 3.19 |
| Slim Green                     | Control (−0.03)                       | 1.29                                                                                        | 4.19 | 9.46 | 0.40  | 1.06 | 2.13 | 3.23                                | 3.95 | 4.44 |
|                                | −0.25                                 | 0.74                                                                                        | 2.04 | 3.51 | 0.34  | 0.63 | 1.09 | 2.18                                | 3.24 | 3.22 |
|                                | −0.50                                 | 0.31                                                                                        | 0.71 | 1.06 | 0.19  | 0.36 | 0.39 | 1.63                                | 1.97 | 2.72 |
| Kentucky Wonder                | Control (−0.03)                       | 3.06                                                                                        | 4.94 | 9.44 | 0.87  | 1.23 | 2.40 | 3.52                                | 4.02 | 3.93 |
|                                | −0.25                                 | 1.22                                                                                        | 2.34 | 3.33 | 0.66  | 0.89 | 1.76 | 1.85                                | 2.63 | 1.89 |
|                                | −0.50                                 | 0.55                                                                                        | 0.98 | 2.37 | 0.44  | 0.63 | 0.82 | 1.25                                | 1.56 | 2.89 |
| LSD (0.05) salinity × cultivar |                                       | 0.14                                                                                        | 0.22 | 0.32 | 0.03  | 0.06 | 0.10 | 0.17                                | 0.21 | 0.20 |

Summary of the significance of variance sources

|              |    |    |    |    |    |    |    |    |    |
|--------------|----|----|----|----|----|----|----|----|----|
| Cultivar (C) | ** | ** | *  | ** | *  | ** | *  | ** | *  |
| Salinity (S) | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| C × S        | ** | ** | ** | ** | ** | ** | ** | ** | ** |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.  
<sup>a</sup> Represents the means for pots containing two plants with three replicates.  
<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.  
\*,\*\* Significant at *P* = 0.05 and 0.01, respectively.

Nitrogen-15 contents of green bean shoots were reported [122] as being of higher magnitudes than those in roots, for all three cultivars, at all salinity levels (Table 35.4). These differences are considered to be due to the larger dry weights of shoots than those of roots, for all cultivars (larger sink size). The shoot/root ratios of <sup>15</sup>N content tended to increase with time and decrease with increasing salinity for all cultivars, except for Kentucky Wonder at the third harvest with −0.25 MPa stress. This may have been due to the retarded translocation of <sup>15</sup>N from roots to shoots caused by salt stress and accumulation of <sup>15</sup>N in shoots as the growth period progressed. This observation is also clearly seen by comparing the <sup>15</sup>N concentration values between shoots and roots (Table 35.5, data from Ref. [122]). The concentration of <sup>15</sup>N in both shoots and roots increased as the growth period progressed, and decreased as the salinity level increased for all cultivars. This pattern was similar to the shoot/root ratios of <sup>15</sup>N content of plants. The <sup>15</sup>N concentration in roots was far greater than that in shoots, for all cultivars. This higher concentration of <sup>15</sup>N in roots can be explained, in part, as the absorption of NH<sub>4</sub><sup>+</sup> onto the root surface, or infusion of ammonium and nitrate ions into the root apparent free space, as suggested by Pessarakli and Tucker [135,137,138] and Pessarakli [122].

**35.2.5 NITROGEN-15 UPTAKE RATES**

The <sup>15</sup>N-uptake rate, expressed as mg <sup>15</sup>N absorbed per kg dry matter produced by plants per day, is presented in Table 35.6 (data from Ref. [122]). At each salinity level, <sup>15</sup>N uptake rates peaked at the earliest harvest and decreased as the growth period progressed, for both the shoots and roots in each cultivar. This finding indicates that the younger plants absorbed <sup>15</sup>N at a faster rate than

**TABLE 35.5**  
**Nitrogen (<sup>15</sup>N) Concentration of Plant Parts of Three Green Bean Cultivars**  
**as Affected by Three Levels of NaCl Stress at Three Harvest Times**

|                                                        |                                       | <sup>15</sup> N Concentration of Plant Parts<br>(mg <sup>15</sup> N kg <sup>-1</sup> Dry Weight) <sup>a</sup> |      |      |       |      |      |
|--------------------------------------------------------|---------------------------------------|---------------------------------------------------------------------------------------------------------------|------|------|-------|------|------|
|                                                        |                                       | Harvest <sup>b</sup>                                                                                          |      |      |       |      |      |
| Cultivar                                               | Salt Stress (Osmotic Potential) (MPa) | Shoots                                                                                                        |      |      | Roots |      |      |
|                                                        |                                       | 1                                                                                                             | 2    | 3    | 1     | 2    | 3    |
| Tender Improved                                        | Control (−0.03)                       | 1035                                                                                                          | 1077 | 1182 | 1167  | 1214 | 1291 |
|                                                        | −0.25                                 | 571                                                                                                           | 757  | 1106 | 1015  | 1333 | 1297 |
|                                                        | −0.50                                 | 563                                                                                                           | 808  | 910  | 826   | 1309 | 1140 |
| Slim Green                                             | Control (−0.03)                       | 733                                                                                                           | 988  | 1310 | 1111  | 1359 | 1411 |
|                                                        | −0.25                                 | 561                                                                                                           | 944  | 1000 | 1000  | 1286 | 1239 |
|                                                        | −0.50                                 | 408                                                                                                           | 772  | 791  | 905   | 1125 | 951  |
| Kentucky Wonder                                        | Control (−0.03)                       | 981                                                                                                           | 1141 | 1252 | 1299  | 1447 | 1569 |
|                                                        | −0.25                                 | 731                                                                                                           | 955  | 1047 | 1200  | 1156 | 1375 |
|                                                        | −0.50                                 | 579                                                                                                           | 790  | 983  | 1023  | 1235 | 1139 |
| LSD (0.05) salinity × cultivar                         |                                       | 21                                                                                                            | 25   | 27   | 16    | 18   | 20   |
| <i>Summary of the significance of variance sources</i> |                                       |                                                                                                               |      |      |       |      |      |
| Cultivar (C)                                           |                                       | **                                                                                                            | **   | *    | *     | **   | **   |
| Salinity (S)                                           |                                       | **                                                                                                            | **   | **   | **    | **   | **   |
| C × S                                                  |                                       | **                                                                                                            | **   | **   | **    | **   | **   |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.

<sup>a</sup> Represents the means for pots containing two plants with three replicates.

<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.

\*, \*\* Significant at *P* = 0.05 and 0.01, respectively.

the older ones, regardless of stress level. Nevertheless, <sup>15</sup>N-uptake rates significantly decreased under NaCl stress as compared with the controls, at each harvest, for all cultivars, in both plant parts, except for the roots of the Tender Improved at the second and third harvests. Slim Green shoots, at the earliest harvest, had substantially lower <sup>15</sup>N-uptake rate than the other two cultivars under normal condition.

**35.2.6 PROTEIN SYNTHESIS BY PLANTS**

The crude protein contents of both shoots and roots of the three green bean cultivars were markedly lower under stress conditions as compared with the controls (Table 35.7, data from Ref. [128]). Under stress conditions, the Tender Improved cultivar produced significantly more protein than the other two cultivars. Protein synthesis in shoots was substantially higher than that in roots for all the three cultivars. This significant difference appears to be due to the higher dry matter production of shoots than roots for any treatment for any of the three cultivars. Pessarakli et al. [129] used two sources of N (ammonium and nitrate) for evaluating protein synthesis in green beans and found that, under normal (nonsaline) condition, the nitrate-treated plants synthesized appreciably more protein than the ammonium treated ones, at each harvest, for all three cultivars. This phenomenon was more noticeable in roots than in shoots for each cultivar. However, except for the Tender Improved cultivar at the first harvest, the crude protein content of plants was substantially lower under stress as compared with the controls for either source of N. Salt stress had

**TABLE 35.6**  
**Nitrogen (<sup>15</sup>N) Uptake Rate of Plant Parts of Three Green Bean Cultivars as Affected by Three Levels of NaCl Stress at Three Harvest Times**

|                                                 |                                       | <sup>15</sup> N Uptake Rate of Plant Parts<br>(mg <sup>15</sup> N kg <sup>-1</sup> Dry Weight) <sup>a</sup> |     |    |       |     |     |
|-------------------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------------------------------|-----|----|-------|-----|-----|
|                                                 |                                       | Harvest <sup>b</sup>                                                                                        |     |    |       |     |     |
| Cultivar                                        | Salt Stress (Osmotic Potential) (MPa) | Shoots                                                                                                      |     |    | Roots |     |     |
|                                                 |                                       | 1                                                                                                           | 2   | 3  | 1     | 2   | 3   |
| Tender Improved                                 | Control (−0.03)                       | 207                                                                                                         | 108 | 79 | 233   | 121 | 86  |
|                                                 | −0.25                                 | 114                                                                                                         | 76  | 74 | 203   | 133 | 87  |
|                                                 | −0.50                                 | 113                                                                                                         | 81  | 61 | 165   | 131 | 76  |
| Slim Green                                      | Control (−0.03)                       | 147                                                                                                         | 99  | 87 | 222   | 136 | 94  |
|                                                 | −0.25                                 | 112                                                                                                         | 94  | 67 | 200   | 129 | 83  |
|                                                 | −0.50                                 | 82                                                                                                          | 77  | 53 | 181   | 113 | 63  |
| Kentucky Wonder                                 | Control (−0.03)                       | 196                                                                                                         | 114 | 84 | 260   | 145 | 105 |
|                                                 | −0.25                                 | 146                                                                                                         | 96  | 70 | 240   | 116 | 92  |
|                                                 | −0.50                                 | 116                                                                                                         | 79  | 66 | 205   | 124 | 76  |
| LSD (0.05) salinity × cultivar                  |                                       | 11                                                                                                          | 10  | 7  | 8     | 6   | 5   |
| Summary of the significance of variance sources |                                       |                                                                                                             |     |    |       |     |     |
| Cultivar (C)                                    |                                       |                                                                                                             |     | NS | *     | **  | *   |
| Salinity (S)                                    |                                       | **                                                                                                          | **  | ** | **    | **  | **  |
| C × S                                           |                                       | **                                                                                                          | **  | *  | **    | **  | **  |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.  
<sup>a</sup> Represents the means for pots containing two plants with three replicates.  
<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.  
\*, \*\* Significant at *P* = 0.05 and 0.01, respectively.

the most severe effect on protein synthesis in Slim Green, among the three cultivars, for both NH<sub>4</sub>-N and NO<sub>3</sub>-N sources of N.

The impaired protein synthesis under stress conditions by other bean cultivars such as red kidney beans [62,147] and other types of plants such as barley [75], cotton [136], alfalfa (*Medicago sativa* L.) [127], peas [80], wheat (*Triticum aestivum* L.) [1], tobacco (*Nicotiana tabaccum* L.) [32], corn [110], and soybean [26] have been reported previously by many investigators. In these studies, either decreased amino acid incorporation into protein or the reduction in polyribosome levels due to the salt stress was reported as the reason for the depressed protein synthesis by plants. This may be a reason for reduction in protein synthesis in green beans.

**35.2.7 WATER UPTAKE BY PLANTS**

For all three cultivars of green beans, total water uptake decreased with increased salinity (Table 35.8, data from Ref. [122]), and the decrease patterns were similar to those of dry-matter production [122]. Tender Improved absorbed more water than Kentucky Wonder and Slim Green cultivars under NaCl-stress conditions. However, under normal condition, Kentucky Wonder absorbed significantly more water than the other two cultivars, at the second and third harvests [122]. The absorbed water values for Slim Green were the lowest among the three cultivars, at each harvest, for any corresponding treatment. Reduction in water uptake by other plants, or other bean cultivars, due to salt stress has been reported by many investigators [61,92,97,112,117,118,130,135,137–139,147,156].



**TABLE 35.7**  
**Crude Protein Content of Three Green Bean Cultivars under Various NaCl Stress Levels at Different Harvest Times**

|                 |                                       | Crude Protein Content of Plant Parts <sup>a</sup> (mg) |      |       |       |      |      |
|-----------------|---------------------------------------|--------------------------------------------------------|------|-------|-------|------|------|
|                 |                                       | Harvest <sup>b</sup>                                   |      |       |       |      |      |
| Cultivar        | Salt Stress (Osmotic Potential) (MPa) | Shoots                                                 |      |       | Roots |      |      |
|                 |                                       | 1                                                      | 2    | 3     | 1     | 2    | 3    |
| Tender Improved | Control (−0.03)                       | 361                                                    | 521  | 766   | 92    | 108  | 149  |
|                 | −0.25                                 | 239                                                    | 453  | 653   | 56    | 113  | 165  |
|                 | −0.50                                 | 181                                                    | 314  | 416   | 47    | 101  | 108  |
| Slim Green      | Control (−0.03)                       | 172                                                    | 379  | 866   | 50    | 71   | 180  |
|                 | −0.25                                 | 156                                                    | 260  | 465   | 43    | 51   | 93   |
|                 | −0.50                                 | 63                                                     | 91   | 138   | 27    | 32   | 41   |
| Kentucky Wonder | Control (−0.03)                       | 259                                                    | 350  | 683   | 79    | 89   | 256  |
|                 | −0.25                                 | 142                                                    | 187  | 332   | 61    | 68   | 157  |
|                 | −0.50                                 | 103                                                    | 161  | 295   | 46    | 51   | 66   |
| LSD (0.05)*     |                                       | 43.2                                                   | 61.5 | 101.4 | 16.8  | 18.3 | 35.1 |

Source: Pessarakli, M. et al., *J. Plant Nutr.*, 12(10), 1105, 1989a.  
<sup>a</sup> Represents the means for pots containing two plants with three replicates.  
<sup>b</sup> Harvests 1, 2, and 3 are for 5, 10, and 15 days <sup>15</sup>N uptake periods, respectively.  
\* Represents the least significant difference between the treatment means at the 0.05 level of confidence.

These investigators generally agreed that plant root permeability (expressed as hydraulic conductivity of the root system) decreased significantly under salt-stress conditions. This may explain the reduction in the water-uptake rate and may contribute to a similar reduction in nutrient absorption, resulting in retarded plant growth and decreased dry-matter production under salt-stress conditions.

**35.2.8 WATER-USE EFFICIENCY OF PLANTS**

Water-use efficiency, expressed as mL water absorbed per g dry matter produced by plants, is exhibited in Table 35.8 (data from Ref. [122]). These data indicate that all three cultivars tended to use water more efficiently at the earliest harvest than at later harvests, either under normal or stress conditions. This appears to be due to the faster rate of growth and higher dry-matter production rate (g dry matter produced per day, which can be calculated from the dry-matter data, Table 35.1) at the earliest harvest than at later harvests (a dilution effect). Nevertheless, all cultivars at each harvest (except, for Slim Green and Kentucky Wonder at the first harvest) used substantially less water for each unit of dry matter produced under stress conditions as compared with the controls. Omami et al. [117] also reported that plants with different salinity tolerance showed different water use efficiency. In Pessarakli's [122] study, at each harvest, for any corresponding treatment (except, control plants at the first harvest), Tender Improved used substantially less water for each unit of dry matter produced (used water more efficiently) than the other two cultivars. Reduced water use efficiency under saline conditions was also reported by Zhang et al. [188]. However, these investigators found that application of mulch was significantly effective in saving water and improving water use efficiency and crop yield under saline irrigation condition. Their results showed that use of mulches significantly reduced ET (improved water use efficiency) of Swiss chard under salinity stress condition imposed by saline irrigation.

**TABLE 35.8**  
**Total Water Absorption and Water Use Efficiency by Three Green Bean Cultivars as Affected by Three Levels of NaCl Stress at Three Harvest Times**

| Cultivar                                               | Salt Stress<br>(Osmotic Potential)<br>(MPa) | Water Uptake<br>(mL H <sub>2</sub> O Pot <sup>-1</sup> ) <sup>a</sup> |      |      | Water Use Efficiency<br>(mL H <sub>2</sub> O g <sup>-1</sup> Dry Weight) <sup>a</sup> |      |      |
|--------------------------------------------------------|---------------------------------------------|-----------------------------------------------------------------------|------|------|---------------------------------------------------------------------------------------|------|------|
|                                                        |                                             | Harvest <sup>b</sup>                                                  |      |      |                                                                                       |      |      |
|                                                        |                                             | 1                                                                     | 2    | 3    | 1                                                                                     | 2    | 3    |
| Tender Improved                                        | Control (−0.03)                             | 2035                                                                  | 4160 | 6010 | 514                                                                                   | 676  | 689  |
|                                                        | −0.25                                       | 1310                                                                  | 3275 | 4800 | 384                                                                                   | 610  | 602  |
|                                                        | −0.50                                       | 800                                                                   | 2125 | 3325 | 336                                                                                   | 478  | 584  |
| Slim Green                                             | Control (−0.03)                             | 1085                                                                  | 2985 | 5685 | 513                                                                                   | 1057 | 1150 |
|                                                        | −0.25                                       | 840                                                                   | 2005 | 3555 | 506                                                                                   | 757  | 815  |
|                                                        | −0.50                                       | 485                                                                   | 1310 | 2010 | 500                                                                                   | 596  | 652  |
| Kentucky Wonder                                        | Control (−0.03)                             | 1800                                                                  | 4315 | 7840 | 500                                                                                   | 1019 | 905  |
|                                                        | −0.25                                       | 1085                                                                  | 2225 | 3525 | 490                                                                                   | 834  | 865  |
|                                                        | −0.50                                       | 690                                                                   | 1780 | 2830 | 475                                                                                   | 692  | 792  |
| LSD (0.05) salinity × cultivar                         |                                             | 86                                                                    | 118  | 143  | 42                                                                                    | 47   | 61   |
| <i>Summary of the significance of variance sources</i> |                                             |                                                                       |      |      |                                                                                       |      |      |
| Cultivar (C)                                           |                                             | **                                                                    | **   | **   | NS                                                                                    | *    | **   |
| Salinity (S)                                           |                                             | **                                                                    | **   | **   | *                                                                                     | **   | **   |
| C × S                                                  |                                             | **                                                                    | **   | **   | *                                                                                     | **   | **   |

Source: Pessarakli, M., *Crop Sci.*, 31(6), 1633, 1991.

<sup>a</sup> Represents the means for pots containing two plants with three replicates.

<sup>b</sup> Harvests 1, 2, and 3 are for 5-, 10-, and 15-d <sup>15</sup>N-uptake periods, respectively.

\*, \*\* Significant at *P* = 0.05 and 0.01, respectively.

35.3 SUMMARY AND CONCLUSIONS

Effects of NaCl stress on dry-matter production, total N, <sup>15</sup>N, crude protein, and water uptake by three green bean cultivars were discussed in this chapter.

Total dry-matter production was greater for Tender Improved than for Kentucky Wonder and Slim Green cultivars, for any corresponding treatment, at each harvest. For all three cultivars, total dry weight decreased significantly with increasing salinity. Reduction in dry weight due to NaCl stress was less for Tender Improved than for the other two cultivars. Total N and <sup>15</sup>N uptake, by all three cultivars, substantially decreased under NaCl stress conditions. Nitrogen-15 concentration and shoot/root ratios of <sup>15</sup>N decreased with increasing salinity. Nitrogen-15 concentrations of shoots were less than those of roots, for all plants. Sodium chloride stress severely reduced the crude protein content of plant parts for all three cultivars, at all three harvests. However, the Tender Improved appears to be less affected by salinity than the other two cultivars. Shoots of all plants contained substantially higher total crude protein than roots for all treatments. This appears to be due to the higher biomass of shoots than roots for any corresponding treatment. Nevertheless, shoots were more severely affected than roots by salinity when salinized plants were compared with the controls for each plant part. Sodium chloride stress severely decreased the crude protein content of all three cultivars at each harvest for both sources of <sup>15</sup>N. However, the Tender Improved appeared to be the least and the Slim Green the most severely affected by salinity among the three cultivars.

Under normal (nonsaline) conditions, green beans appear to absorb and utilize more  $\text{NO}_3\text{-N}$  than  $\text{NH}_4\text{-N}$  into protein synthesis. In contrast, under salt stress,  $\text{NO}_3\text{-N}$  seems more severely affected than  $\text{NH}_4\text{-N}$  for being incorporated into protein. Furthermore, any level of salt stress will likely cause a drastic reduction in protein content and N metabolism in the salt-sensitive bean plants.

For all cultivars, water uptake also was substantially decreased under stress conditions, particularly, at the highest level of stress. Among the three cultivars, Tender Improved was the least and Slim Green the most severely affected by salinity in all aspects of stress. This is an indication of the difference in the salt tolerance of these cultivars. Therefore, among the three cultivars discussed here, Tender Improved cultivar of green beans appears the most suitable for growing under field conditions. Furthermore, since there are numerous cultivars of green beans, additional testing of their response under saline conditions could detect a wider range of tolerance and susceptibility to soil salinity. This will enable researchers to select the most salt-tolerant cultivars to be recommended to the growers.

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# 36 Physiology and Molecular Biology of the Effects of Salinity on Rice

*R.K. Singh and T.J. Flowers*

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## 36.1 INTRODUCTION

The majority of past studies of abiotic stress tolerance have compared the physiological status of a stressed plant with that of an unstressed control plant in order to deduce underlying mechanisms. In general, these studies have not included the molecular and genetic bases of stress tolerance to support the physiological findings. However, recent developments in the field of genetics and molecular biology have opened up exciting new possibilities in understanding the physiology of abiotic stresses (Bennett and Khush, 2003; Eynard et al., 2005; Ismail et al., 2007). Almost all the abiotic stresses—drought, submergence, salinity, alkalinity, toxicities of Fe and Al, and deficiencies of P and Zn—limit rice production especially in the rainfed environments that contribute about half of the global rice area (Gregorio et al., 2002; Lafitte et al., 2004). Among these stresses, salinity has a particularly long-lasting effect on plant productivity as, unlike the situation with drought that can be relieved by irrigation, it is extremely difficult to remove salts from agricultural soils. In this chapter, we summarize some of the recent advances in understanding the molecular biology underlying the response of rice to salinity and sodicity and provide updated information particularly on quantitative trait loci associated with salt tolerance in rice.

### 36.1.1 WHY DO WE NEED SALT-RESISTANT GENOTYPES?

Although world cereal production increased from 0.9 billion tons in 1960 to 2 billion tons in 2006 (FAO, 2006) and rice production has increased about threefold from 200 million tons in 1960 to about 635 million tons in 2006, the area of land cultivated for rice has not increased substantially (Maclean et al., 2002; FAO, 2006). About 90% of the world's rice is produced in Asia—the continent, where the crop is the most important staple food, is home to most of the world's poor and has one of the world's highest population growth rates. Since there is little possibility for any expansion of the area for rice production in most Asian countries, the challenge to feed the rapidly increasing population is daunting. To produce more rice, three options remain: expand the extent of irrigation in those areas or favorable environments with a potential for high productivity; increase the productivity of rice in unfavorable ecosystems; and harness areas so far unfavorable for food production. Achieving the first option is unlikely because of the increasing competition for irrigation water from urban and industrial users. For the second and third options, the availability of high-yielding and abiotic stress-tolerant crop varieties is imperative. Hence, new breeding efforts for abiotic stress-tolerant cultivars need to be initiated and existing programs strengthened and sustained. The enormous, potentially negative, impact of climate change on production of the world's major food crops, including rice (Wassmann et al., 2009) will complicate achieving these options. This is particularly so for salinization, where recent estimates in an FAO database indicate more than 800 million hectares of land throughout the world as salt-affected, either by salinity (4.025 million km<sup>2</sup>) or sodicity (4.344 million km<sup>2</sup>) (FAO, 2000).

## 36.2 SALINITY AND SODICITY

In agronomic terms, salinity is generally defined as the presence of sufficient concentrations of soluble salts in the soil to reduce normal crop growth. The excess salts are most commonly chlorides and sulfates of sodium and magnesium. Salinity is measured in terms of the electrical conductivity (EC) of a soil extract: an electrical conductivity of more than 4 dS m<sup>-1</sup> indicates a saline soil (Table 36.1, USSL Staff, 1954; Eynard et al., 2005). Sodic soils are characterized by a concentration of Na<sup>+</sup> of more than 15% at the exchange sites of the negatively charged clay

**TABLE 36.1**  
**Diagnostics of Salt-Affected Soils**

| Salt-Affected Type | Electrical Conductivity <sup>a</sup><br>(EC <sub>e</sub> ) (dS m <sup>-1</sup> ) | Exchangeable Sodium<br>Percentage <sup>a</sup> (ESP) (%) | pH <sub>1:2</sub> <sup>b</sup> |
|--------------------|----------------------------------------------------------------------------------|----------------------------------------------------------|--------------------------------|
| Saline             | >4                                                                               | <15                                                      | <8.8                           |
| Sodic              | <4                                                                               | >15                                                      | 8.5–10.7                       |
| Saline-sodic       | >4                                                                               | >15                                                      | Variable                       |

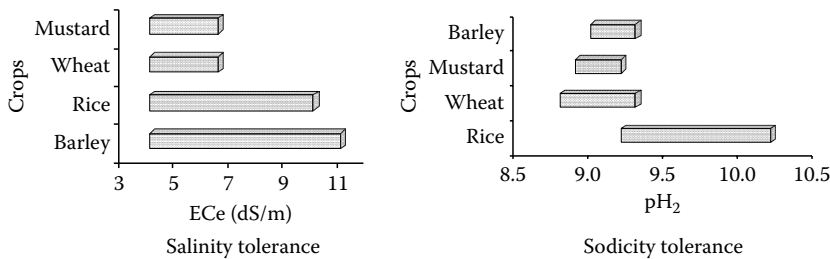
<sup>a</sup> At 25°C.  
<sup>b</sup> pH<sub>1:2</sub>—pH of soil solution containing one part of soil and two parts of water.

particles and a high exchangeable Na<sup>+</sup> percentage (ESP), which raises the pH of a saturated soil extract to be more than 8.5. Such soils are characterized by an excess of carbonates and bicarbonates of Na<sup>+</sup> and low hydraulic conductivity. Saline-sodic soils behave both as saline and sodic soils.

### 36.3 LIMITS OF THE TOLERANCE OF RICE WITH RESPECT TO OTHER CROPS

Plants vary in their salt tolerance from the halophytes that grow in seawaters (Flowers and Colmer, 2008) to those such as chickpea that are sensitive to one-tenth or less of the seawater salt concentration (Flowers et al., 2009): most crop species hardly tolerate a quarter to a third of the concentration of salts found in seawater (Flowers, 2004). Salinity affects the growth of plants in numerous ways: morphologically, physiologically, and biochemically. The tolerance of salt is a complex trait, both genetically and physiologically and is the sum affect of different contributory mechanisms, most of which are governed by polygenes (Moeljopawiro and Ikehashi, 1981; Mishra, 1996; Mishra et al., 1998; Singh et al., 2001). The discovery of quantitative trait loci (QTLs) associated with salt tolerance in various crops like tomato, wheat, barley, and rice, support this hypothesis (Mano and Takeda, 1997; Ma et al., 2007; Cheng et al., 2008; Läuchli et al., 2008; Witcombe et al., 2008; Zang et al., 2008).

Maas and Hoffman (1977) divided crops into four categories (tolerant, moderately tolerant, moderately sensitive, and sensitive) based on the response of yield to increasing salinity and categorized by a threshold value at which a response was observed and the rate at which yield declined with increasing salinity. Rice was categorized as sensitive by Maas and Hoffman (1977) and yet, rice has a large variability for tolerance of salinity (Flowers and Yeo, 1981; Lisa et al., 2004; Mahmood et al., 2004; Zeng et al., 2004; Munns et al., 2006) as well as sodicity (Mishra, 1996). For example, the two genotypes Pokkali (highly tolerant of salinity) and IR29 (extremely sensitive) show extreme differences in their response to salinity. Hence, to categorize rice as a sensitive crop may not be truly meaningful in terms of the possibilities of enhancing its resistance to salinity, especially as rice gene banks hold more than 100,000 accessions from around the world. Variability is the foundation of plant breeding and intra-crop variability available for a desired trait indicates the probability of success: the greater the intra-crop variability, the greater the promise for developing the desired trait. It is also important to distinguish between the response to salinity and alkalinity: rice has a great deal of tolerance to alkalinity and much less to salinity; barley, on the other hand, is highly tolerant to salinity and has little resistance to alkalinity (Figure 36.1). Rice is an effective crop for the reclamation of sodic soils (Singh, 1994) and, because of its tolerance to flooding and its range of salinity tolerance, can be used in cases of coastal salinity (Lafitte et al., 2004; Singh et al., 2009).

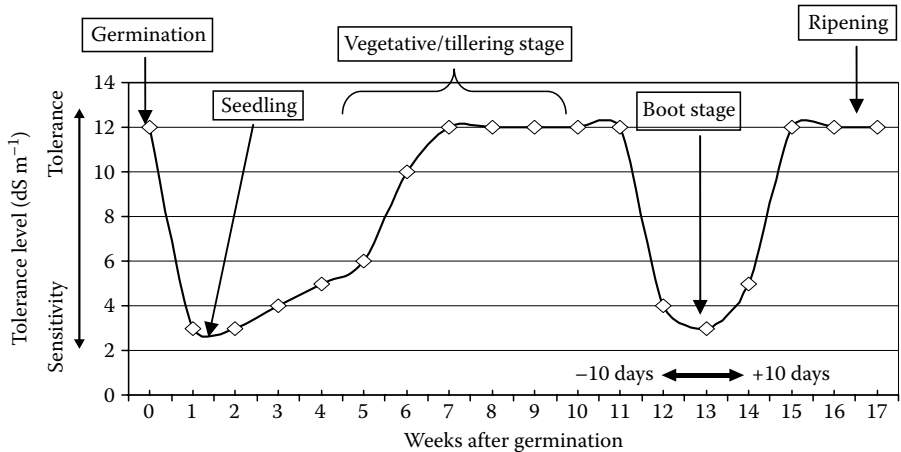


**FIGURE 36.1** Range of variability for salinity/sodicity tolerance in important crops. The floating bars denotes the existence of intra-crop variability for salinity or sodicity while the lower limits of bars indicates that all the varieties tested below this limit were almost unaffected by the salt stress (ECe or pH<sub>2</sub>). (Adapted from Mishra, B., *Highlights of Research on Crops and Varieties for Salt Affected Soils*, CSSRI, Karnal, India, 1996.)

**36.4 EFFECTS OF SALINITY ON VEGETATIVE GROWTH AND YIELD**

The threshold salinity at which the yield of rice is affected can be as low as 3 dS m<sup>-1</sup> (Maas and Hoffmann, 1977). However, this sensitivity to salt not only varies between genotypes as previously mentioned, but between stages of plant development, as depicted in Figure 36.2 for a typical 120-day rice variety. Germination is relatively tolerant, but growth becomes very sensitive during the early seedling stage (1–3 weeks), gains tolerance during active tillering, but becomes sensitive during panicle initiation, anthesis, and fertilization, and finally is relatively more tolerant at maturity (Khatun and Flowers, 1995a; Lutts et al., 1995; Makihara et al., 1999; Singh et al., 2004; Shereen et al., 2005).

Germination of rice is surprisingly resistant to salt, given the sensitivity of yield. For example, in an experiment conducted to assess the germinability of various rice varieties using a single salt (NaCl) at a concentration of 1.6% (~28 dS m<sup>-1</sup>) and a mixture of salts commonly found in salinized soils in India (containing MgCl<sub>2</sub>, NaCl, CaCl<sub>2</sub>, and Na<sub>2</sub>SO<sub>4</sub> in a ratio of 1:4:5:10). Germination of some varieties occurred at more than double sea water salinity (Sankar et al., 2006): such concentrations are far beyond those tolerated during vegetative growth. In another experiment (Agnihotri et al., 2006), land races of rice could germinate in 200 mM NaCl (~20 dS m<sup>-1</sup>), which again is a higher concentration than that in which rice will grow. However, studies have shown



**FIGURE 36.2** Variation in the sensitivity of rice to salinity during its ontogeny. (From Singh, R.K. et al., *J. Indian Soc. Coastal Agric. Res.*, 26, 16–21, 2008. With permission.)

a very poor correlation between tolerance at the seedling stage with that during reproduction, suggesting that salinity affects a different set of genes at these two stages (Moradi and Ismail, 2007; Rao et al., 2008). The rice genotype FL478 (IR66946-178-3R-1-1-1) is a very good example of the difference in tolerance at different growth stages. This recombinant inbred line (RIL) from an IR29/Pokkali cross is extremely tolerant at the seedling stage (and is now being used as the salt-tolerant check in place of Pokkali for the seedling stage salinity tolerance studies at IRRI), but if grown under salinity until maturity shows very high spikelet sterility even at 6–8 dS m<sup>-1</sup> (unpublished observation of the first author). The reproductive stage is crucial as it ultimately determines grain yield, but the importance of the seedling stage cannot be underestimated as it determines crop establishment.

### 36.4.1 VEGETATIVE GROWTH

Sodium and chloride ions are the main causes of salt damage to rice, as for other plants. There is a multitude of papers, too many to cite here, showing the sensitivity of the growth of rice to salinity with some genotypes being killed by just 50 mM NaCl over a period of just 2 weeks (e.g., Flowers and Yeo, 1981). NaCl affects plants by altering the water potential in the soil (the osmotic effects of the salt) and through the specific toxicity of the ions. That these ions bring about osmotic and specific toxic effects on plants is well documented (see Munns and Tester, 2008 for references) and it has been argued that these effects are sequential. Plants first respond to the change in water potential and later to the toxicity of the ions involved (Wilson et al., 1970; Munns et al., 1995; Munns and Tester, 2008); osmotic and ionic tolerances are not necessarily related (Munns and Termaat, 1986; Munns and Tester, 2008).

The threshold salinity at which growth of rice begins to be affected by the salt can be as low as 3 dS m<sup>-1</sup> (~30 mM salt). In general, such low salt concentrations are unlikely to cause medium or long-term osmotic stress (Yeo, 2007). Osmotic stress has a direct and immediate effect on plant growth (Wilson et al., 1970). However, this phase of response is over relatively quickly in rice; growth recovers (not necessarily to the original rate) over a period of 24 h, so any osmotic effects are transient. (Yeo et al., 1991; Roshandel and Flowers, 2009) and there is little time when osmotic stress can be equated with drought stress (cf. Munns et al., 1995). How any osmotic stress immediately signals to down-regulate plant growth is not entirely clear, but ABA signaling and changes in stomatal conductance are the probable candidates. For plants such as rice that are sensitive to relatively low salt concentrations, the osmotic effects are likely to be outweighed by specific toxic effects of the ions on enzyme activity (Bhandal and Malik, 1988; Yeo, 2007) or processes such as protein synthesis (Flowers and Dalmond, 1992; Blaha et al., 2000) or by effects such as the reduction of nutrient uptake (e.g., K<sup>+</sup>).

While it is clear that seedling or early vegetative growth is very sensitive to salinity, there is a large genetic variation in response with many land races in particular found to be relatively tolerant. Consequently, traditional land races such as Pokkali and Nona Bokra have often been used as donors of tolerance in breeding programs; other land races and improved genotypes found to be tolerant to a salinity of 12 dS m<sup>-1</sup> at the seedling stage are Cheriviruppu (IRGC 19928), Kalimekri 77-5 (IRTP 14213), TKM6 (IRTP 11703), Bhura Rata (IRGC 28590), Mushkan 41 (IRGC 6828), Kalarata 1-24 (IRGC 26913), Bhirpala (IRGC 37015), IR4630-22-2-5-1-3 (IRGC 72958), Kajalsail, IR69502-6-SRN-3-UBN-1-B, IR65483-118-25-31-7-1-5, IR65483-141-2-4-4-2-5, IR77298-14-1-2, IR63262-AC201-1-7-2, and IR73689-76-2 (Adorada et al., 2005). (IRTP and IRGC numbers are the accession numbers in IRRI germplasm resource units and gene banks.) However, care must be taken in the use of land races in breeding as they evolved over centuries so that many variations exist among the collections of the same land race. Very often only one of number of accessions of the same land race is tolerant. For example, the IRRI germplasm collection has about a dozen variants of Pokkali and not all of them are tolerant

(so recently, if Pokkali is used without any specific accession number in research programs at IRRI, it is understood that this is accession 108921).

### 36.4.2 YIELD

Surprisingly, for such an important crop as rice, there are few studies that address the effects of salinity on yield. Most research has been limited to the seedling or early vegetative stages or only reports parameters such as fresh or dry weight although the ultimate aim has been to increase grain yield with limited resources keeping the environment intact (Lee et al., 2006b, 2007; Morsy et al., 2006; Tajbakhsh et al., 2006; Chen et al., 2007; Moradi and Ismail, 2007; Singh et al., 2007a; Cheng et al., 2008; Jain et al., 2008; Kanneganti and Gupta, 2008; Zang et al., 2008). Few studies have continued to the reproductive stage and quantified the effect of salinity on yield. These scarce reports vary in their inferences on the effect of salinity on grain yield, probably because of the use of different genotypes. For example, Zeng et al. (2002) showed big varietal differences in salinity tolerance and found IR63731-1-1-4-3-2 to be much more tolerant at both the seedling and reproductive stage than M202. Grain yield per plant declined in linear fashion with increased salinity and as did the harvest index of M202 (Zeng and Shannon, 2000). Mahmood et al. (1999) reported average yield declined by 30% compared to normal soils under salinity ( $10\text{ dS m}^{-1}$ ) using six rice varieties (NR-1, IR 6, NIAB 6, KS 282, Basmati 370 and Basmati 385). They also reported huge varietal differences and found NIAB 6 the most tolerant. Mishra et al. (2000) reported the development of a series of salt-tolerant varieties (CSR10, CSR13, CSR27) that yielded more than 3–4 tonnes  $\text{ha}^{-1}$  in saline ( $\text{EC } 6\text{--}9\text{ dS m}^{-1}$ ) and sodic ( $\text{pH } 9.4\text{--}9.7$ ) soils. CSR10, grown as an intercrop, yielded 4–5 tonnes  $\text{ha}^{-1}$  under moderate sodic stress in large-scale field experiments (Dagar et al., 2001). Thus, it is essential to name the genotype or variety when describing the degree of salinity tolerance or grain yield reduction. Rao et al. (2008) categorized the effect of salinity as tolerant, moderately tolerant, and sensitive when grain yield was reduced by 27%, 46%, and 50% respectively at an  $\text{ECe } 8\text{ dS m}^{-1}$ . Similarly, for sodicity, at pH 9.8 the grain yield reduction was 25%, 37%, and 68% in the three groups, respectively (Rao et al., 2008).

### 36.4.3 MORPHOLOGICAL EFFECTS

The most appropriate question here is which traits are not affected by salt-stress? Although the degree of morphological alteration depends upon the intensity of the salt-stress, the first symptom of salinity in rice is burning of the leaf tip. Other morphological parameters such as low tillering, leaf scorching, spikelet sterility, reduced number of florets per panicle, stunted plant growth, low 1000 grain weight, low grain yield, altered flowering duration, leaf rolling under low RH conditions, and patchy growth at field level are major symptoms of salinity.

### 36.4.4 PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS

Crop varieties and breeding lines differ in their inherent capability to modify various physiological and biochemical processes in response to salt stress. Though numerous physiological and biochemical changes take place under stress, it is thought that only a few changes influence overall salt tolerance. These changes control the solute and water balance of the plant. Particularly, important traits for determining salt tolerance in rice are

- The extent of  $\text{Na}^+$  transport to shoot.
- The extent of  $\text{Cl}^-$  uptake.
- Preferential accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in older leaves and leaf sheaths.
- The ability to maintain  $\text{K}^+$  uptake in the presence of external  $\text{Na}^+$ .
- The ability to maintain P and Zn uptake.
- The ability to synthesize non-toxic organic compatible solutes.



## 36.5 MECHANISM OF TOLERANCE TO SALT STRESS IN RICE

### 36.5.1 AVOIDANCE

Although rice is particularly sensitive to salinity at the seedling stage (see Khatun and Flowers, 1995a), being a transplanted crop, it is possible to alleviate stress through management by transplanting aged seedlings. Although it is generally not possible to avoid stress at the flowering stage, it is sometimes possible to use early maturing genotypes to avoid terminal salinity that can occur under coastal saline conditions. For example, this is a common practice in the coastal areas of Bangladesh and India (Orissa and West Bengal) especially in the dry (*boro*) season. In any analysis of the response of genotypes to salinity it is important that attributes associated with avoidance should be carefully separated from those associated with genetically based tolerance.

### 36.5.2 INITIAL ENTRY OF SALTS FROM ROOTS

Plants roots are the first parts of the plant to experience any soil-based salt stress and it is at the roots that the entry of  $\text{Na}^+$  and  $\text{Cl}^-$  is determined. The phenomenon by which plants minimize the entry of toxic ion through their roots is called “salt exclusion” (see Colmer et al., 2006). Comparing the external and internal (xylem) concentration of  $\text{Na}^+$ , it has been estimated that most plants “exclude” about 98% of the salt in the soil solution; only about 2% is allowed to be transported to shoot through xylem (Munns, 2005). Some “good” wheat varieties “exclude” up to 99% of saline ions; rice, although salt-sensitive, still “excludes” about 94% of soil  $\text{Na}^+$  from its transpiration stream (Munns, 2005; Munns et al., 2006). Interestingly, barley, the most tolerant of the cereal crops “excludes” a similar proportion of the external sodium to rice (Munns, 2005), but at much higher salt concentrations suggesting it is better able to deal with accumulated salt than is rice.

#### 36.5.2.1 $\text{Na}^+$ and $\text{Cl}^-$

$\text{Na}^+$  can clearly enter into roots along with the water stream which moves from soil to the vascular system either by a symplastic or an apoplastic pathway. Any symplastic transport requires movement through plasma membranes before offloading to the xylem. Apoplastic transport, also called bypass flow occurs when ions move through cell walls and other extracellular spaces to the xylem. Such apoplastic pathways are commonly checked by physical barriers such as Casparian strips: discontinuities in such barriers are thought to allow direct access of solution to the stele. In rice roots, bypass flow has been shown to be a significant part of sodium entry under saline conditions (Yeo et al., 1987; Yeo, 1992; Yadav et al., 1996; Garcia et al., 1997; Yeo et al., 1999; Ochiai and Matoh, 2002; Anil et al., 2005; Gong et al., 2006; Krishnamurthy et al., 2009). About 30% of  $\text{Na}^+$  reaching the shoots of hydroponically grown IR36 (in 50 mM NaCl) has been estimated to reach the shoots by bypass flow (Faiyue et al., 2010a). The magnitude of bypass flow in rice is thought to depend on the anatomical and morphological developments of the roots (Yeo et al., 1987; Yeo, 1992; Yadav et al., 1996; Garcia et al., 1997; Gong et al., 2006; Krishnamurthy et al., 2009). However, our understanding of the specific roles of particular roots in bypass flow is still poor. Recent research using lateral rootless mutants (*lrl1*, *lrl2*), a crown rootless mutant (*crl1*), their wild types (Oochikara, Nipponbare, and Taichung 65, respectively) and seedlings of the rice cv. IR36 suggests that the path of bypass flow in rice does not include the sites of lateral root emergence (Faiyue et al., 2010b), although lateral roots do probably play an important part in the apoplastic entry of ions in hydroponically grown rice (Faiyue et al., 2010a). Using the bypass flow tracer, trisodium-8-hydroxy-1,3,6-pyrenetrisulfonic acid (PTS) with the rice cv. IR36, PTS was identified in the vascular tissue of the lateral roots using both epifluorescence microscopy and confocal laser scanning microscopy (Faiyue et al., 2010a). Cryo-scanning electron microscopy and epifluorescence microscopy of sections stained with berberine-aniline blue showed that an exodermis was absent in the lateral roots suggesting that the lack of this barrier allowed PTS to move freely through the cortical layers, enter

the stele, and be transported to the shoots via transpiration stream. Both silicon and polyethylene glycol are able to reduce apoplastic transport in rice (Yeo et al., 1999; Gong et al., 2006; Faiyue et al., 2010a), presumably by blocking pores in the cell walls. It is the high concentration of ions in the apoplast (see Flowers et al., 1991) that leads to one of the early symptoms (see Section 36.4.3) of salinity, leaf tip burning. This occurs when water is transpired, leaving high concentrations of salt in the apoplast, which causes osmotic water withdrawal from neighboring cells.

The way in which  $\text{Na}^+$  is taken up by plant cells remains uncertain and to some extent controversial (Tester and Davenport, 2003; Wang et al., 2007; Chen et al., 2009; Zhang et al., 2010). For symplastic transport, where uptake of  $\text{Na}^+$  involves a transporter, the situation is quite uncertain as there are a number of proteins capable of transporting  $\text{Na}^+$  and  $\text{K}^+$  (Tester and Davenport, 2003; Maathuis, 2007; Zhang et al., 2010). The transporters commonly thought to play a role in low affinity uptake are nonselective cation channels (NCSCs), high-affinity  $\text{K}^+$  transporters (HKTs),  $\text{K}^+$  uptake permeases/high-affinity  $\text{K}^+/\text{K}^+$  transporters (KUP/HAK/KT), cation-chloride co-transporters (CCCs) and possibly members of the Shaker family of  $\text{K}^+$  transporter (AKT) (Flowers and Colmer, 2008; Zhang et al., 2010). The uncertainty is, in part, a consequence of technical problems associated with the investigation of ion transport at high external concentrations. While uptake of ions from the concentrations found in normal agricultural soils can be followed by a range of techniques (see, e.g., Tester and Davenport, 2003), at high external salt concentrations the use of gene knockout is compromised by pleiotropic effects and that of heterologous expression by the failure of such systems to reproduce kinetic characteristics of transporters *in planta* (see, Wang et al., 2007). A further important factor in interpreting experimental data is the conditions under which the experiments were performed. Thus for rice, where experiments are carried out in external concentrations above about 50 mM NaCl, it may not be possible to distinguish between a response that is adaptive and one that is pathological.

High external concentrations of  $\text{Na}^+$  interfere with the uptake of  $\text{K}^+$  and it is a common finding that  $\text{K}^+$  concentrations decline as do  $\text{K}^+/\text{Na}^+$  ratios under saline conditions (Zhang et al., 2010): this is certainly the case for rice, where the genes determining the uptake of the two ions appear to be located on different linkage groups (Koyama et al., 2001; Lin et al., 2004). Consequently, the maintenance of  $\text{K}^+/\text{Na}^+$  ratios is an important contributory trait in salt tolerance. Differential regulation of the membrane proteins' transcripts in the tolerant and sensitive genotypes is the main determinant for the ultimate phenotypic expression. The up-regulation of *OsCHX11* in FL478 (a tolerant rice) enhance the cultivar's ability to maintain a significantly higher  $\text{K}^+$  concentration in its tissues compared IR 29 (Senadheera et al., 2009). A reduction in  $\text{Na}^+$  transport from root to shoot is also a desirable trait. Ren et al., (2005) cloned a rice QTL (*SKCI*), which encodes an HKT-family member involved in the transport of  $\text{Na}^+$  by unloading it into the xylem sap. This QTL was identified from a salinity-tolerant *indica* rice land race "Nona Bokra." Seedlings carrying the transporter from Nona Bokra were more tolerant than those carrying the same QTL from a sensitive variety and consequently shoot  $\text{Na}^+$  was lower in former than latter group of seedlings, while reverse was the case for  $\text{K}^+$  content (Lin et al., 2004).

$\text{Na}^+$  transport is, in general, unidirectional from root to shoot; however, there are reports of  $\text{Na}^+$  recirculation from shoots to roots through phloem (Munns et al., 1986; Berthomieu et al., 2003). In fact,  $\text{Na}^+$  recirculation is thought to be small (Munns and Tester, 2008) and it is possible to interpret any recirculation as evidence of a failure of sensitive plants to exclude  $\text{Na}^+$  from the phloem rather than as a mechanism of tolerance, i.e., as a pathological response, rather than any adaptive mechanism (see, e.g., Flowers et al., 1986). In rice,  $\text{Na}^+$  recirculation from shoots to roots, as shown by the use of radio-labeled  $\text{Na}^+$ , is insignificant (Yeo and Flowers, 1982) and is greater in the mutant *lrl1* than its wild-type, a finding consistent with the view that that salt-sensitive genotype simply could not keep  $\text{Na}^+$  out of its phloem (Faiyue et al., 2010a).

Although more emphasis is generally placed on the uptake and transport of  $\text{Na}^+$  than of  $\text{Cl}^-$  (probably because of ease of measurement),  $\text{Cl}^-$  is likely quite as toxic as  $\text{Na}^+$  and therefore of equal importance in studies of salinity. Little is known, however, of the means by which  $\text{Cl}^-$  enters

plant cells. The general view is that  $\text{Cl}^-$  uptake across the plasma membrane is effected by symport with  $\text{H}^+$  while vacuolar accumulation occurs via a cation channel (see Munns and Tester, 2008). For rice, Nakamura et al. (2006) isolated cDNA clones *OsCLC-1* and *OsCLC-2* homologous to a tobacco gene that encodes a voltage-gated chloride channel. The proteins from both genes were localized in the vacuolar membranes and expression of *OsCLC-1* increased on treatment with NaCl. Transcriptional regulation during salt stress has been compared in the salt-sensitive  $\text{Cl}^-$ -accumulating rice line IR29 and the salt-tolerant  $\text{Cl}^-$ -excluding rice line Pokkali (Diedhiou and Golldack, 2006). In response to salt stress, *OsCLC1* transcript levels were repressed in leaves and roots of IR29 whereas in Pokkali expression was transiently induced. Under the same conditions, in IR29 mRNA levels of the  $\text{Na}^+/\text{H}^+$  antiporter *OsNHX1* and of the vacuolar  $\text{H}^+$ -ATPase subunit *OsVHA-B* decreased upon salt stress whereas Pokkali showed transient stimulation of *OsVHA-B* transcripts (Diedhiou and Golldack, 2006).

### 36.5.3 DEALING WITH ACCUMULATED SALT

Once accumulated,  $\text{Na}^+$  and  $\text{Cl}^-$  have to be accommodated in vacuoles if they are not to damage the metabolic machinery of cytoplasm. However, there is evidence of variation in rice not only of the ability to accumulate ions in older rather than younger leaves, but also in a differential ability to tolerate ions in cells known as tissue tolerance (see Section 36.5.3.3).

#### 36.5.3.1 Plant Level Transport of Salt and Its Compartmentation

Rice accumulates high concentrations of  $\text{Na}^+$  (and  $\text{Cl}^-$ ) ions in its shoots, which are not evenly distributed: the older leaves accumulate higher concentrations of  $\text{Na}^+$  (and  $\text{Cl}^-$ ) than younger leaves (Yeo and Flowers, 1982, 1986) in spite of having lower transpiration rates than the younger leaves (Yeo et al., 1985). The more tolerant genotypes are better at maintaining low ion concentrations in their younger leaves for longer than are the more sensitive genotypes (Yeo and Flowers, 1986). Exactly how this is achieved is still unclear, but plant vigor can serve to decrease net concentrations.

#### 36.5.3.2 Vigor

Some of the most tolerant rice genotypes are the most vigorous, typified by the non-dwarfed land races such as Pokkali and Nona Bokra. Dwarfing reduces vigor and hence the biomass into which accumulated salts can be sequestered. In an analysis of the physiological traits contributing to salt tolerance in rice, vigor was found to explain a greater proportion of the variance than  $\text{Na}^+$  concentration or tissue tolerance (Yeo et al., 1990). Direct selection for vigor and yield has already been proved useful in rice under drought conditions (Venuprasad et al., 2007; Kumar et al., 2008; Serraj et al., 2009).

#### 36.5.3.3 Tissue Tolerance

Tissue tolerance is the ability of tissue to tolerate accumulated  $\text{Na}^+$  and  $\text{Cl}^-$ . It presumably reflects the ability to compartmentalize toxic ions and maintain  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations as low as 10–30 mM within the cytoplasm (Carden et al., 2003), although the exact concentration at which the cytosolic activities are inhibited is not well defined. The highest tolerable concentration is thought to lie somewhere between 50 and 100 mM, beyond which many enzymes are inhibited (Flowers, 1972; Greenway and Osmond, 1972; Flowers and Dalmond, 1992; Flowers and Colmer, 2008; Munns and Tester, 2008). Where vacuolar salt concentrations are higher than those tolerated in the cytoplasm, osmotic adjustment is achieved through the accumulation of compatible solutes. So, tissue tolerance is likely to be an amalgamation of these distinct processes.

Estimation of tissue tolerance can be a tedious process. For rice, this has been achieved by measuring the salt concentration in leaf tissue that is associated with the loss of 50% of the chlorophyll. Practically, this requires salinizing seedlings and then after a period of time, estimating chlorophyll and  $\text{Na}^+$  (or  $\text{Cl}^-$ ) concentration in the same tissue sample. So, in order to look at

variation in tissue tolerance between genotypes, measurement of  $\text{Na}^+$  and chlorophyll on at least three replicates of 50 plants of each genotype is required (Yeo and Flowers, 1983). In spite of the difficulties, variation in tissue tolerance has been reported for rice (Yeo et al., 1990). It is not easy to use this variation in any breeding program, however, and a molecular marker for the trait is clearly needed.

#### 36.5.3.4 Intracellular Compartmentation of the Toxic Ions

Sequestration of  $\text{Na}^+$  (and  $\text{Cl}^-$ ) in vacuoles is the way in which the low cytosolic ion concentrations are maintained by plants. Ion homeostasis is governed by the proton pumps and ion transporters located on plasma and tonoplast membranes. The proton ATPases of these membranes together with the pyrophosphatase of the tonoplast generate the thermodynamic gradients by which  $\text{Na}^+/\text{H}^+$  antiporters and  $\text{Cl}^-/\text{H}^+$  symporters facilitate the movement of  $\text{Na}^+$  and  $\text{Cl}^-$  from the cytosol to the apoplast or the vacuole.  $\text{Na}^+$  that enters the cell from the soil/cell wall interface, perhaps via a nonselective cation channel although this is by no means certain (see Section 36.5.2.1), is thought to be removed to the apoplast (cell wall/soil or cell wall/xylem) via a  $\text{Na}^+/\text{H}^+$  antiporter known as salt overly sensitive 1 (SOS1) (see Fig. 3, Munns and Tester, 2008). Sequestration of  $\text{Na}^+$  to the vacuole is effected by a different  $\text{Na}^+/\text{H}^+$  antiporter, NHX. There is evidence for SOS1 and NHX in rice.

A functional homolog of the arabidopsis SOS1 has been identified in rice (Martinez-Atienza et al., 2007) as has another putative plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter, *OsNHAI*, which is up-regulated under salt stress (Zhou et al., 2006). However, the role of  $\text{Na}^+/\text{H}^+$  antiport in salt tolerance is unclear as return of  $\text{Na}^+$  to the cell wall does not remove ions from the rhizosphere, but simply serves to exacerbate their concentration adjacent to the plasmalemma. It is possible that SOS1 functions in xylem loading of  $\text{Na}^+$  (Munns and Tester, 2008), where enhancing its activity would aid removal of ions from roots for sequestration in the shoots.

A gene *OsNHX1* encodes a  $\text{Na}^+/\text{H}^+$  antiporter in rice (Fukuda et al., 1999, 2004) although the protein also appears to facilitate the exchange of other monovalent cations (Kinclova-Zimmermannova et al., 2004). There is some evidence that over-expression of *NHX1* enhances salt tolerance in rice (Chen et al., 2007) as does transformation with the NHX genes from other plant species (Ohta et al., 2002; Fukuda et al., 2004; Zhao et al., 2006a,b,c; Verma et al., 2007).

#### 36.5.3.5 Synthesis of Osmoprotectants

Once  $\text{Na}^+$  is sequestered into the vacuole, the synthesis of soluble but metabolically inactive compound in the cytoplasm balances the osmotic potential across the tonoplast. These osmoprotectants or compatible solutes can be amino acids (e.g., proline), quaternary compounds (e.g., glycine betaine), sugars (e.g., sucrose), or sugar alcohols (e.g., mannitol). They are postulated to be present in the cytoplasm at osmotically active concentrations (Flowers et al., 1977; Bohnert et al., 1995; Munns and Tester, 2008) although the evidence for their cytoplasmic localization is sparse (see Flowers and Colmer, 2008). It would clearly be advantageous for a plant if the synthesis of osmoprotectants were governed by the pleiotropic control of vacuolar  $\text{Na}^+/\text{H}^+$  antiporter activity.

Rice does not synthesize glycine betaine (Rathinasabapathi et al., 1993). Although rice has one of the necessary enzymes (betaine aldehyde dehydrogenase, BADH) (Nakamura et al., 1997), it lacks a functional choline monooxygenase (CMO), the second enzyme required for the synthesis of glycine betaine (Luo et al., 2007). Rice transformed with BADH from barley and fed exogenous glycine betaine shows increased salt tolerance (Kishitani et al., 2000). Others have also shown exposure to exogenous glycine betaine can enhance tolerance to salt (Harinasut et al., 1996; Demiral and Turkan, 2004) and drought (Farooq et al., 2008). Transgenic rice expressing the *CMO* gene from spinach has been produced and is claimed to show enhanced tolerance to salt (Shirasawa et al., 2006). Transgenics transformed to express choline oxidase (*codA*), a bacterial gene that can induce the synthesis of glycine betaine in rice (Sakamoto et al., 1998; Mohanty et al., 2002) and to drought (Sawahel, 2003); some increase in tolerance was claimed for rice expressing a bacterial choline oxidase (Su et al., 2006).

Among the other compatible solutes, rice accumulates proline under saline conditions (e.g., Lin and Kao, 1996; Lin et al., 2002; Kumar et al., 2008), but it is not clear whether this is of adaptive value or part of the pathology of salt injury. Increased tolerance to salinity has been claimed for rice expressing a bacterial mannitol dehydrogenase (Pujni et al., 2007). Recently, there has been growing interest of utilization of trehalose metabolism to ameliorate the effects of abiotic stresses. Trehalose, a nonreducing sugar, protects biological molecules from desiccation damage. Garg et al. (2002) have demonstrated the expression of trehalose biosynthesis in rice-conferred tolerance to multiple abiotic stresses. The increase in trehalose levels in transgenic rice lines of Pusa Basmati 1 (PB1) using either tissue-specific or stress-dependent promoter, resulted into a higher capacity for photosynthesis and concomitant decrease in the extent of photooxidative damage during salt, drought, and low-temperature stresses. However, the concentrations of trehalose were too low for the molecule to act as an osmoprotectant, and it is possible that trehalose acts through a signaling cascade (Flowers, 2004).

### 36.5.3.6 Up-Regulation of Antioxidants

Salt stress and other abiotic stresses generate a secondary oxidative stress caused by the accumulation of reactive oxygen species (ROS). ROS include hydrogen peroxide, hydroxyl radicals, and superoxide anions. While ROS are generated under normal cellular activity such as photorespiration and  $\beta$ -oxidation of fatty acids, their concentrations increase under biotic and abiotic stresses. Under normal growth conditions, the production of ROS in cells is as low as  $240\mu\text{M s}^{-1}$  superoxide and the steady-state level of  $\text{H}_2\text{O}_2$  in chloroplast is  $0.5\mu\text{M}$  (Polle, 2001; Mittler, 2002). However, under salinity, the level of ROS production reaches as high as  $720\mu\text{M s}^{-1}$  (a threefold increase) and  $\text{H}_2\text{O}_2$  level can be as high as  $15\mu\text{M}$  (a 30 fold increase). It is reported that a  $\text{H}_2\text{O}_2$  concentration of  $10\mu\text{M}$  reduces the net photosynthesis rate by 50%. Superoxide and  $\text{H}_2\text{O}_2$  toxicity have been attributed to a cascade of reactions that result into the production of hydroxyl radicals and other destructive species such as lipid peroxidases that damage vital macromolecules by protein denaturation, mutation, and lipid peroxidation (Vaidyanathan et al., 2003).

The capacity of plants to scavenge ROS and to reduce their damaging effects appears to represent an important stress-tolerance trait. Elimination of ROS is achieved either by antioxidant compounds such as glutathione, thioredoxin, ascorbate, and carotenoids or by ROS-scavenging enzymes such as superoxide dismutase, catalase, glutathione peroxidases, and peroxiredoxins. Accumulation of  $\text{H}_2\text{O}_2$  has been demonstrated in rice (Wi et al., 2006) and rice genotypes appear to differ in the activities of enzymes associated with scavenging ROS (Vaidyanathan et al., 2003; Demiral and Turkan, 2005; Moradi and Ismail, 2007). Genes encoding enzymes associated with scavenging ROS are up-regulated during salt stress (Moons, 2003; Menezes-Benavente et al., 2004; Hong et al., 2007, 2009a,b).

Another group of compounds, polyamines, are also reported to have antioxidant activities. Accumulation of the three main polyamines putrescine, spermidine, and spermine play a role in stress tolerance (osmotic stress, salinity, ozone, UV). The polycationic nature of polyamines, positively charged at physiological pH, has led to the hypothesis that these sites could bind to anionic sites associated with nucleic acids and membrane phospholipids. Besides being involved in numerous cellular functions, polyamines are free-radical scavengers and act as antioxidants although their exact mode of action is not yet fully understood (Groppa and Benavides, 2008). Rice genotypes accumulate polyamines (Basu et al., 1988; Basu and Ghosh, 1991; Bay et al., 1992) and while it has been reported that foliar application of putrescine can mitigate the effects of salinity (Krishnamurthy, 1991) in other experiments neither putrescine (Lin and Kao, 1995) nor spermidine appeared to be related to salt tolerance (Maiale et al., 2004). Contrary to this, greater accumulation of spermidine and spermine is reported in salt-tolerant than salt-sensitive varieties, associated with the conversion from their precursor (putrescine). High spermidine and spermine contents are associated with cell membrane systems and tolerant varieties have more stable membranes than sensitive ones (Bay et al., 1992). However, the degree to which polyamines accumulate

is a function of the nitrogen status of the plants (Yamamoto et al., 2004), which may explain differences between earlier reports. There is some evidence that transformation of rice with arginine decarboxylase (ADC) led to increased polyamine accumulation and enhanced salt tolerance (Roy and Wu, 2001).

### 36.5.3.7 Signaling Pathways: Transcription Factors

Stress-related transcription factors bind to specific sequences of the promoter regions of target genes that need to be activated, collectively or sequentially, in response to that stress, playing key roles in the coordination of the plant's reaction. Different families of transcription factors have been identified, such as CBF/DREBs (C-repeat/dehydration-responsive element binding factors), NAC (NAM, ATAF, CUC) family, Hsfs (heat shock factors), and SAP (stress-associated protein) (Cao et al., 2006; Ito et al., 2006; Hu et al., 2008, 2009; Kanneganti and Gupta, 2008; Wang et al., 2008; Zheng et al., 2009).

Rice DREB homologs (*OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D*, and *OsDREB2A*) have been studied for their expression. *OsDREB1A* and *OsDREB1B* were induced by cold, whereas expression of *OsDREB2A* was induced by dehydration and high-salt stresses (Dubouzet et al., 2003). Over-expression of *OsDREB1A* in transgenic arabidopsis-induced over-expression of target stress-inducible genes resulting in plants with higher tolerance to drought, high salt, and freezing stresses (Dubouzet et al., 2003; Ito et al., 2006). This indicated that *OsDREB1A* has functional similarity to arabidopsis *DREB1A*. The structures of DREB1-type ERF/AP2 domains in monocots are closely related to each other as compared with that in the dicotyledonous plants. *OsDREB1A* is potentially useful for producing transgenic monocots that are tolerant to drought, high salt, and/or cold stresses (Dubouzet et al., 2003; Oh et al., 2005; Ito et al., 2006). The DREB transcription factor, *OsDREB1F*, has been cloned and characterized from rice (Wang et al., 2008). Expression analysis revealed that the *OsDREB1F* gene was induced by most of the abiotic stresses (salt, drought, cold) and also by ABA application, but not by pathogens, wounding, or H<sub>2</sub>O<sub>2</sub>. Subcellular localization results indicated that *OsDREB1F* is a nuclear gene that encodes a transcription activator that can specifically bind to DRE/CRT (G/ACCGAC) but not to ABRE (ABA responsive elements). Transgenic rice plants with the *OsDREB1F* gene had enhanced tolerance to salt, drought, and low temperature (Wang et al., 2008) and over-expressing *OsDREB1D* in arabidopsis enhanced its salt tolerance (Zhang et al., 2009b). Constitutive over-expression of the genes encoding these proteins can induce the constitutive expression of many genes resulting in increased tolerance, but associated with reduced growth even under non-stressed conditions. *DREB1A* activated at least 12 genes in arabidopsis (Seki et al., 2001) but caused dwarfism of the plants although expressing the arabidopsis genes *CBF3/DREB1A* (*CBF3*) in rice enhanced salt tolerance but did not stunt growth (Oh et al., 2005). Hence, such genes, when used with a stress-inducible promoter (*rd29A*), generate plants with a normal appearance and showed enhance stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000).

The NAC genes are members of one of the biggest families of plant-specific transcription factors. There are 75 putative NAC genes in rice and 105 in arabidopsis (Ooka et al., 2003; Gao et al., 2007). *ONAC045* has been functionally characterized for its response to abiotic stresses (Riechmann et al., 2000). Expression analysis revealed that *ONAC045* was induced by drought, high salt, and low-temperature stresses, and abscisic acid (ABA) treatment in leaves and roots. Transcriptional activation assays in yeast indicated that *ONAC045* is a transcriptional activator; it is also localized in the nucleus. Transgenic rice plants over-expressing *ONAC045* showed enhanced tolerance to drought and salt treatments. When the transgenic rice seedlings were treated with 200 mM NaCl, the expression of *ONAC045* was highly induced in roots after 4, 12, and 24 h of NaCl treatment but just slightly induced in leaves (Zheng et al., 2009). Another NAC transcription factor, *OsNAC6*, has been found activated early after exposure to salt (Nakashima et al., 2007). This gene is also induced by cold, salt, drought, and ABA (Chao et al., 2005; Ohnishi et al., 2005).

Two other stress-responsive genes *OsLEA3-1* and *OsPM1* when over-expressed increased stress tolerance (Zheng et al., 2009). As mentioned above, transgenic plants over-expressing some stress-responsive genes, such as *OsNAC6/SNAC2* (Nakashima et al., 2007; Hu et al., 2008), *OsDREB1A*, and *OsDREB1B* (Ito et al., 2006) show growth retardation under normal condition that may finally result in a yield penalty; however, no growth or yield penalty was observed in any one of the non-stressed transgenic rice over-expressing *ONAC045* under greenhouse conditions (Zheng et al., 2009).

### 36.5.3.8 Stress-Activated Protein Pathways

Rice, like other plants, produces many stress-responsive proteins induced by heat, cold, salt, or drought (Chitteti and Peng, 2007; Liu et al., 2007; Malakshah et al., 2007; Hu, 2008; Huang et al., 2008a,b; Hu et al., 2009; Zhang et al., 2009a) and there have been numerous analyses of the effects of salt on the proteome of rice (e.g., Moons et al., 1995; Salekdeh et al., 2002; Abbasi and Komatsu, 2004; Akino et al., 2005; Kim et al., 2005; Yan et al., 2005; Dooki et al., 2006; Parker et al., 2006; Chitteti and Peng, 2007; Malakshah et al., 2007; Zang and Komatsu, 2007; Chen et al., 2009; Cheng et al., 2009; Song et al., 2009). As with studies of changes of gene expression, as an environmental factor is changed, the concentrations of many proteins will change relative to other proteins and over time. While the magnitude of such changes were interesting *per se* in early studies, it was difficult to determine their significance and to separate adaptive from pathological effects. We now need hypothesis-driven analyses of such changes; for example, the analysis of how the synthesis of members of the LEA family of proteins function during desiccation/salt stress in rice, as suggested by Moons et al. (1995). Evidence also implicates a putative osmo-sensory histidine kinase (AtHK1)-MAPK cascade and its negative regulators (AtMKP1) in salt-stress signaling that probably leads to osmotic homeostasis and ROS (reactive oxygen species) scavenging (Chinnusamy and Zhu, 2003; Martinez-Atienza et al., 2007). Another kind of protein that responds to abiotic stresses are molecular chaperones (also called heat shock proteins, Hsps) that functions in protein restructuring, protein intracellular localization, and secretion. Heat shock factors (Hsfs) are the transcriptional activators of Hsps. It has been reported that Hsps and Hsfs are widely involved in response to various abiotic stresses such as heat, drought, salinity, and cold. Most Hsfs and Hsps have highly similar and overlapped response and regulation patterns under different stresses as reported earlier; however, some of those genes show significantly specific response to distinct stress (Hu et al., 2009).

## 36.6 GENETICS OF SALT TOLERANCE

The mode of inheritance and heritability are the most important factors that determine success in plant breeding; they embrace the mode of gene action, association of different contributing traits due to linkage or pleiotropy, and genetic expression that is an interplay with the environment ( $G \times E$  interaction). Initial reports suggested the role of a few dominant genes for the inheritance of some of the contributing traits for salt tolerance in rice (Akbar et al., 1972; Akbar and Yabuno, 1975, 1977), but subsequent studies showed the continuous variation in the segregating generation indicating polygenic inheritance for salt tolerance and its contributing traits (Moeljopawiro and Ikehashi, 1981; Mishra et al., 1998; Singh et al., 2001). Maternal effects could not be realized but there was little skewness in the distribution of the segregating progenies, suggesting the role of a few major genes along with numerous minor genes in the inheritance of salt tolerance (Mishra et al., 1998; Singh et al., 2001).

Diallel studies in rice have revealed the preponderance of both additive and non-additive gene action for almost all the characters associated with salt tolerance (Narayanan and SreeRangasamy, 1991; Gregorio and Senadhira, 1993; Mishra 1996; Gregorio et al., 2002; Chauhan, 2007). Expression of the dominant components for grain yield is usually suppressed under stress. This suggests that to generate salinity-tolerant varieties, donors with more additive genes for grain

yield should be used. As far as narrow-sense heritability ( $h^2_{ns}$ ) is concerned, there have been different findings. Gregorio and Senadhira (1993) reported high environmental effects with low narrow-sense heritability estimates for low  $Na^+/K^+$  ratio in a  $9 \times 9$  diallel in rice, while heritabilities of about 0.4–0.5 were reported for two segregating breeding lines for  $Na^+$ ,  $K^+$ , and  $Na^+/K^+$  ratio by Garcia et al. (1997). Gupta (1999) also reported a high magnitude of narrow-sense heritability for  $Na^+/K^+$  ratio for both alkali (0.66) and saline soils (0.63) and for grain yield. Chauhan (2007) reported narrow-sense heritabilities of 0.71, 0.76, 0.65, 0.74, and 0.71 for plant height in normal, moderate sodic (pH  $\sim$  9.4), high sodic (pH  $\sim$  9.6), moderate saline ( $EC_e \sim 4 \text{ dS m}^{-1}$ ), and high saline ( $EC_e \sim 6 \text{ dS m}^{-1}$ ) environments, respectively. Other characters that showed high narrow sense heritability were panicle length (0.61), days to flowering (0.55) and 100 seed weight (0.54) in normal; panicle length (0.63), days to flowering (0.57) and 100 seed weight in moderate sodic; panicle length (0.56), yield per plant (0.54) and days to flowering in high sodic; days of flowering (0.54), yield per plant (0.51) and panicle length (0.49) in moderate saline; and yield per plant (0.62), days to flowering (0.52) and panicle length (0.52) in high saline environments. Low narrow-sense heritability was observed for yield per plant (0.20) in normal, tillers per plant (0.27) in moderate sodic, 100 seed weight (0.3) in high sodic, tillers per plant (0.2) in moderate saline, and 100 seed weight (0.22) in high saline environment. If the narrow-sense heritability is low, early-generation population size should be large; replications and locations over years should be increased, if possible, to screen the right genotypes, and selection should be done in the later generations. If a trait associated with salt tolerance has high narrow-sense heritability, then early selection could lead to the development of better salt-tolerant rice varieties. However, Garcia et al. (1995) concluded that early selection for agronomic traits should be made after selection for physiological traits such as leaf  $Na^+$  concentration and, ideally, be delayed until the population has reached near-homozygosity. Large  $G \times E$  interactions for yield and yield components have been reported across the range of test environments (Mishra 1996; Chauhan, 2007; Manneh et al., 2007).

Direct selection under stress for grain yield has been reported as the best approach to breed stress-tolerant rice varieties (Mishra 1996; Munns et al., 2006; Venuprasad et al., 2007; Kumar et al., 2008), but selection for the component traits to breed a stress-tolerant rice variety have been proposed (Yeo et al., 1990) and long used (Singh et al., 2004, 2008; Serraj et al., 2009). Morphological traits such as the proportion of filled/unfilled grains, grains per panicle, spikelet fertility, plant height, fertile tillers, and flowering in comparison to non-stress are good indicators associated with salt tolerance in rice. However grain yield is negatively correlated with physiological traits like  $Na^+$  concentration and  $Na^+/K^+$  ratio, whereas it had a positive correlation with  $K^+$  concentration under both alkali and saline soil conditions (Chauhan, 2007; Rao et al., 2008).

### 36.7 MOLECULAR MARKERS: QTL FOR SALT TOLERANCE

Identifying a closely linked robust molecular marker for a target gene or quantitative trait locus (QTL) is the most desirable step forward toward marker-assisted breeding. The more tightly linked the marker for the QTLs, the less the chances of crossing over during recombination and so the higher the probability of marker and trait of interest being inherited together. A gene-based marker is ideal as in the case of *SUB1* and submergence tolerance because the presence of the molecular marker in the population ensures the gene's carryover over generations (Xu et al., 2006). A general aim is to identify specific morphological or physiological traits in diverse cultivated or wild germplasm that might be expected to contribute to improved performance under stress and to transfer these to otherwise adapted varieties through marker-aided pyramiding of the underlying alleles (Gale, 2003; Neeraja et al., 2007). Singh et al. (2007b) summarized the reports on QTL for salinity tolerance in rice starting from Claes et al. (1990) report on *salT* locus on chromosome 1 that was responsible for "Jacalin-like lectin domain protein" of 146 amino acids. The review extended to the



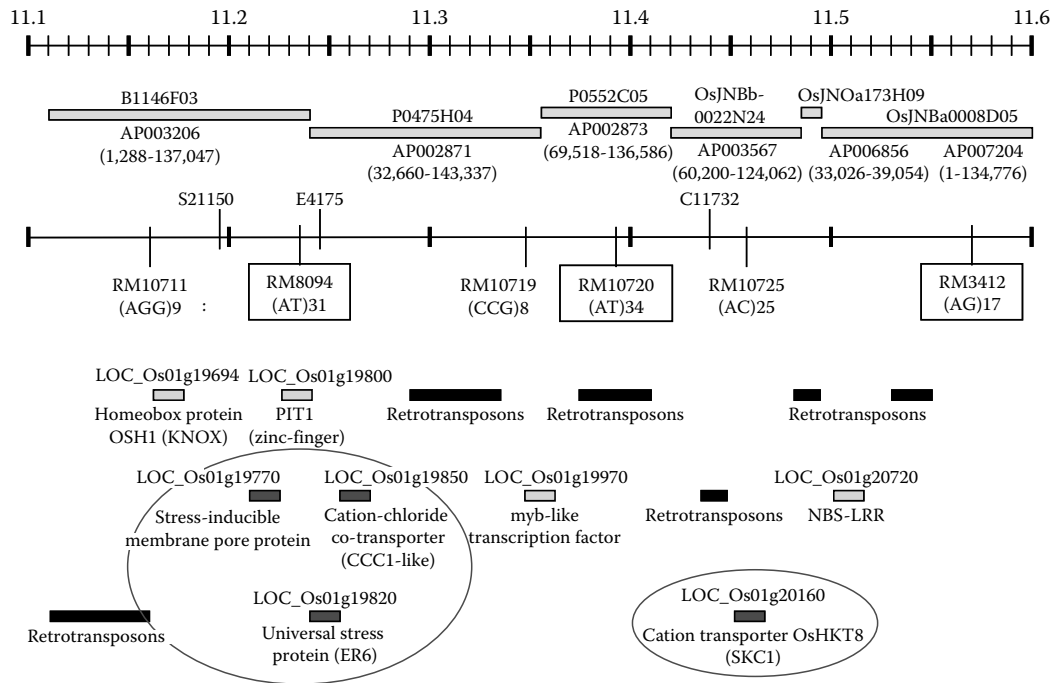
QTL work on seedling stage salinity tolerance of Lee et al. (2006a). Here, we have updated this chapter in Appendix 36.A with several more recent studies.

A major gene for salt tolerance was mapped on chromosome 7 involving RFLP probes using an  $F_2$  population derived from a salt-tolerant *japonica* rice mutant, M-20 and the sensitive original variety 77-170 (Zhang et al., 1995). A major QTL designated “*Saltol*” was mapped on chromosome 1 using a RIL population generated from a cross between the sensitive variety IR29 and a tolerant landrace, Pokkali. This QTL was responsible for  $\text{Na}^+$  and  $\text{K}^+$  absorption as well as  $\text{Na}^+/\text{K}^+$  ratio and each accounted for more than 60% of the variation in this population (Gregorio, 1997; Bonilla et al., 2002). Early studies reported the QTLs responsible for physiological parameters conferring seedling stage salinity tolerance in rice on different chromosomes (Flowers et al., 2000; Koyama et al., 2001; Lang et al., 2001), and substantiated the independence of  $\text{Na}^+$  and  $\text{K}^+$  uptake as they were located on different linkage groups. Some of the studies have shown co-localization of QTLs for  $\text{Na}^+$ ,  $\text{K}^+$ , and/or their ratios on chromosome 1 (Gregorio, 1997; Koyama et al., 2001; Niones, 2004) and chromosome 12 (Lang et al., 2001). Indeed,  $\text{Na}^+/\text{K}^+$  ratio is a derived trait but a balance of both ions is very important within cytosol; thus it indicates that uptake of both the ions either could be due to the linkage or pleiotropic effects within the same gene complex that is quite desirable (where the QTLs for different traits are co-localized) or could be due to probable epistatic interactions (where QTLs for the different traits are on separate linkage group). Prasad et al. (2000) identified seven QTLs for seedling traits associated with salt stress, which were mapped to five different chromosomes. Although they each explained about 15% of the phenotypic variation, none had LOD score of even 3. This was probably due to non-involvement of any salt-tolerant parent in the double haploid population used for study.

So far, the most systematic study for QTL identification and map-based cloning of genes responsible for salt tolerance was the identification of *qSKC1* controlling  $\text{K}^+/\text{Na}^+$  homeostasis under salt stress and encodes an OsHKT-type transporter (Lin et al., 2004; Ren et al., 2005). Nona Bokra was used as the salt-tolerant donor and the population was derived from an *indica/japonica* (Koshihikari) cross. *SKC1* (Os01g20160) is located within the *Saltol* locus. This is a large-effect QTL with LOD score of 11.7 and explained more than 40% of the phenotypic variation (Lin et al., 2004). Ren et al. (2005) reported the further progress of map-based cloning of *qSKC1*. They identified a NIL with a very small Nona Bokra introgression involving *SKC1* region in the Koshihikari background that had substitution of 6 nucleotides. The substitution is responsible for the altered protein by 4 amino acids, which probably is responsible for the functional difference of SKC1 from Nona Bokra and Koshihikari. Two very closely flanking CAPS (cleaved amplified polymorphic sequence) markers, K159 and K061, were developed to identify *SKC1*. Takehisa et al. (2004) used backcrossed inbred lines (BILs) and RFLP markers in a field study to identify the QTLs for traits contributing to salinity tolerance and found them on chromosomes 3 and 7 under saline conditions. The same group also studied leaf bronzing in response to salinity and located *qLB3* with a LOD score of more than 30, which explained more than 80% of phenotypic variation (Takehisa et al., 2004). *Japonica/indica* crosses have quite frequently been used in molecular studies as they give good parental polymorphism. Such an *indica/japonica* cross (Milyang 23/Gihobyee) was used to identify QTLs for salinity tolerance at the seedling stage and showed one QTL on chromosome 1 with a large effect and another on chromosome 3 (Lee et al., 2006a,b). Zang et al. (2008) used 99 introgression lines ( $\text{BC}_2\text{F}_8$ ) derived from another *indica/japonica* cross (IR64 and Binam). A probability of  $<0.005$  was used as the threshold to declare the presence of a QTL in one-way ANOVA instead of LOD scores and phenotypic variation. Both parents were only moderately tolerant of salinity, but the introgression lines that were evaluated showed great variation in salinity tolerance. The authors agreed that during recombination, some hidden favorable genes recombined to generate tolerance—an argument presented nearly 20 years previously by Yeo et al. (1990). QTLs identified for both seedling and tillering stages could be used to pyramid genes into a single agronomic background to enhance the level of salt tolerance.

Not all recent analyses have used *indica/japonica* crosses. An *indica/indica*  $F_{2:3}$  population involving Tarommahali, a tolerant genotype and Khazar, a sensitive genotype, was used to detect the QTLs for multiple traits, mostly physiological. The LOD values ranged from around 3–5 with an explained variation ranging from 9 ( $Na^+/K^+$  ratio) to as high as 38% ( $K^+$  uptake) (Sabouri and Sabouri, 2008; Sabouri et al., 2009). Ammar et al. (2009) also used a  $F_{2:3}$  mapping population derived from an *indica/indica* cross of CSR27 (tolerant) and MI-48 (sensitive) to identify QTLs for different parameters contributing to salt tolerance. Seventeen parameters, including seedling salt injury index and  $Na^+$ ,  $K^+$ , and  $Cl^-$  concentration in the leaf and stem tissues at vegetative and reproductive stages, were analyzed. A total of 25 QTLs were detected on chromosomes 1, 2, 3, and 8 that explained the >10% phenotypic variation; 18 QTLs with LOD values greater than 3 have been included in Appendix 36.A. The  $Na^+/K^+$  ratio was controlled by 7 QTLs explaining phenotypic variance in the range of 42%–52%. Four markers recorded multiple QTLs for more than one salt-tolerance parameter that could be controlled by common or tightly linked genes. Most of studies have been limited to seedling stage tolerance but this study reported 3 QTLs explaining large variation for the reproductive stage parameters. So far, the QTLs for salinity tolerance or associated mechanisms have been reported on all of the chromosomes. There is only one QTL reported on chromosome 5 (Yao et al., 2005), but this study involving Iranian genotypes included quite a few putative QTLs on chromosome 5.

Fine mapping of the Saltol QTL is in progress at IRRI using several sets of NILs derived from the cross IR29  $\times$  Pokkali. Marker saturation of the region has incorporated more than 30 SSRs



**FIGURE 36.3** Physical map on the short arm of chromosome 1 between 11.1 and 11.6Mb showing polymorphic SSR markers and four genes targeted for indel marker development: a stress-inducible membrane pore protein, universal stress protein ER6, cation-chloride co-transporter, and *SKC1*. (From Thomson, M.J. et al., QTL mapping and marker-assisted backcrossing for improved salinity tolerance in rice, in *Proceedings of BioAsia 2007: Sixth Asian Crop Science Association Conference and Second International Conference on Rice for the Future*, Bangkok, Thailand, 2007, pp. 6–12. With permission.)

from the IRGSP and custom-designed insertion/deletion (indel) markers at gene loci across the QTL peak region from 10.7 to 12.5 Mb (Thomson et al., 2007). Four major genes, especially for transporters and membrane/stress proteins within Saltol region as annotated using genome browser of erstwhile online TIGR rice genome project (which is now moved to Michigan State University (MSU) and can be browsed using MSU Rice Genome Project (<http://rice.plantbiology.msu.edu/>), and an additional nearby gene (*salT* at 13.8 Mb) are targeted for the development of gene-based PCR markers (encircled in Figure 36.3). The availability of a large number of gene-based markers or fine-mapped QTLs underlying salinity-tolerance component traits could help in pooling of different tolerance mechanisms to enhance the level of salt tolerance in agronomically superior-adapted varieties using MAS.

## 36.8 GENOTYPING VERSUS PHENOTYPING

About a decade ago, genotyping was considered the biggest hurdle for the molecular dissection of traits. But, with the emergence of draft rice sequence (Goff et al., 2002; Yu et al., 2002) and micro-satellite or SSR markers (Akagi et al., 1996; Chen et al., 1997; Temnykh et al., 2000, 2001), genotyping has become easier. The International Rice Genome Sequencing Project (IRGSP) saturated 389 MB of the rice genome with 18,828 SSR markers with a coverage of 95% that gives enormous choice for markers throughout the genome (IRGSP, 2005). Currently, SNP platforms have enabled the high-throughput genotyping using chips of various capacities and the cost per data point has come down drastically.

While genotyping has developed both in precision and efficiency, phenotyping has not progressed at the same speed. In rice, seedling-based protocols for phenotyping (salinity screening) are still quite robust and repeatable but those based on adult plants or the late growth stages are still not precise and repeatable. This is the major reason why most studies are based on seedling-stage tolerance rather than the adult or reproductive stages. Where large-scale phenotyping has been setup, it is very expensive, cumbersome, and sometimes not repeatable.

### 36.8.1 CHALLENGES IN PHENOTYPING FOR SALT STRESS

Phenotyping for salinity tolerance based on morphological parameters is relatively easy at the seedling stage as it can be carried out in solution culture (Gregorio et al., 1997). The Yoshida solution is the most appropriate for rice growth, with  $\text{Na}^+$  replaced by  $\text{K}^+$  (Yoshida et al., 1976; Flowers and Yeo, 1981; Yeo et al., 1985; Singh and Mishra, 2004). However, maintaining rice plants in solution culture to maturity is problematic because it is difficult to provide adequate support. This can be overcome using an automatic circulatory system.

Apart from mechanical support, the timing of the application of the stress is important as the tolerance of plants, including rice, depends upon the growth stage (Makihara et al., 1999; Moradi et al., 2003; Shereen et al., 2005; Rao et al., 2008; Singh et al., 2008). Seedlings of 4–7 days old can be evaluated for their tolerance within 15 days although very young seedlings are particularly sensitive to salt. Once rice plants become older than 2–3 weeks, their tolerance increases, even that of sensitive genotypes. However, rice becomes very sensitive at the boot stage, which corresponds to micro-sporogenesis—stress during this critical stage reduces pollen fertility (Khatun and Flowers, 1995b). However, it is not just pollen sterility but stigmatic receptivity that ultimately affects the seed set (Khatun and Flowers, 1995a; Khatun et al., 1995). There is good correlation between reproductive stage salinity tolerance and grain yield but not always with seedling stage tolerance (Mishra 1996; Singh et al., 2004). Some reports indicated panicle initiation (PI) as another sensitive stage to salinity (Zeng et al., 2001; Moradi et al., 2003).

### 36.8.2 LEVEL OF STRESS

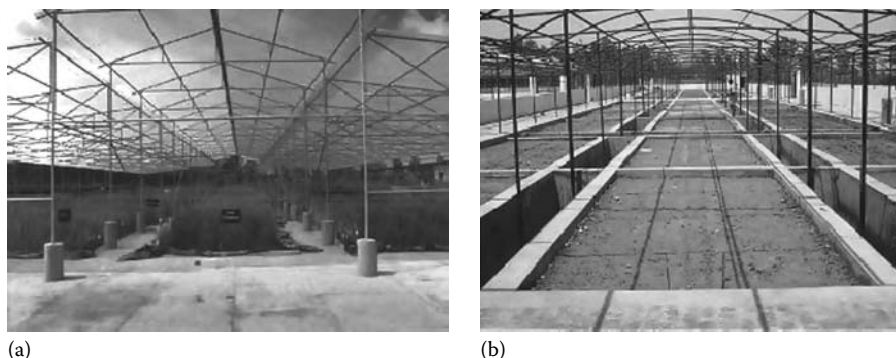
Another question is the level of stress appropriate for phenotyping. Very low salt concentrations may fail to distinguish sensitive and tolerant genotypes, while high stress can kill most genotypes, resulting in poor phenotyping (Flowers and Yeo, 1981). The results of phenotyping also depend upon the ambient condition (temperature and relative humidity). Under high temperature conditions, the rate of transpiration increases carrying more salt into the plant tissues ultimately leading to severe injury or death (Singh et al., 2005). Therefore, the optimum salinity for screening requires adjustment depending upon the ambient conditions. Under controlled conditions (29°C/21°C at 70% RH), 50–120 mM NaCl is adequate to discriminate tolerant and sensitive genotypes of rice at the seedling stage, while 30–100 mM is sufficient for the reproductive stage. Various phenotyping parameters have been used ranging from the morphological (seedling survival—the standard evaluation system or SES score, plant height, maturity, number of tillers, spikelet fertility, pollen viability, and grain yield) to the physiological ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  concentrations,  $\text{Ca}^{2+}$  uptake, the ratio of different ions accumulated during treatment, ionic compartmentation within different tissues) and the biochemical (osmoprotectants, within cell ion compartmentation, ROS, polyamines).

### 36.8.3 STAGE-SPECIFIC SCREENING: SCREENING FOR REPRODUCTIVE-STAGE TOLERANCE

When screening for salinity tolerance at the reproductive stage, the main problem is how to isolate this stage-specific tolerance, i.e., tolerance specifically at the reproductive stage. If the plants are treated at any early stage, even with low salinity (25 mM NaCl), they accumulate salt over time. By the time plants reach the reproductive stage, they may have already accumulated sufficient salt to have reduced their growth. This becomes a biased comparison at the reproductive stage. To address this problem and direct salt to the reproductive stage quickly and efficiently, an experiment was conducted at IRRI by pruning old leaves of rice immediately after the appearance of the flag leaf. The yield data were compared with (1) plants where only the flag leaf remained, (2) plants where the flag leaf and penultimate leaf were left, and (3) plants where the flag leaf and two preceding leaves remained. The experiment indicated that there was no significant effect of leaf pruning on yield except when only the flag leaf remained (1). At this stage, the plants were salinized with 100 mM NaCl for 20 days because 7–10 days before and after the booting is the most sensitive reproductive phase (Figure 36.1). These experimental results greatly increased the efficiency of screening for the reproductive stage salinity tolerance and the mapping population did show distinct differences in their tolerance at this stage. This technique could be used as the basis of reproductive-stage-specific screening for salinity tolerance in rice (Calapit, unpublished data).

Khatun et al. (1995) exposed plants of five rice genotypes of rice (IR54, IR26, IR2153-26-3-5-2, IR 15324-117-3-2-2 and BR6) to salinity (20, 35, or 50 mM  $\text{Na}^+$  in an “artificial seawater”) at panicle initiation in a glasshouse and monitored plant height and dry weight, which were little affected by the treatments. The effects on grain yield were, however, very much more severe than on vegetative growth. The reduction of seed set in crosses suggested that the overall consequences of salinity were dominated by effects on panicle development, stigmas, and grain filling rather than on pollen. There was genotypic variation in  $\text{Na}^+$  accumulation and analysis of the data suggested that genotypic variation exists in the extent to which salinity affects aspects of the plants’ reproductive physiology and development: this variation might be used in attempts to enhance the resistance of rice to salinity.

Screening for salinity tolerance is also possible using microplots (Figure 36.4) filled with soil of specific salinity. Though salinity may vary depending upon the soil moisture, the control of stress



**FIGURE 36.4** Microplots facility at CSSRI Karnal (India) for large-scale adult plant phenotyping until maturity (a) for salinity screening (b) for sodicity screening.

is much better than in natural fields. Plants are transplanted in soil as usual and the salinity of the microplots maintained by saline water irrigation (Mishra, 1996; Singh et al., 2008).

Phenotyping for tolerance to sodicity is more complex than screening for salinity tolerance as it is difficult to produce a solution culture which mimics the natural field sodic conditions due to the insolubility of micronutrients at high pH. Seedling stage sodicity tolerance has been evaluated using a TRIS buffered solution (Singh et al., 2002) but it is not feasible to continue to the adult stage. The major advantage of the buffer-based seedling screening is the uniform stress imposition of stress and potential for high throughput. However for screening adult plants, artificial soil preparation in pots or microplots (Figure 36.4) or in natural fields provide the best option for phenotyping (Singh and Mishra, 2004). The main disadvantage of natural field is spatial variability.

### 36.9 PHYSIOLOGICAL APPROACH FOR IMPROVING TOLERANCE TO HIGH SALT STRESS

To date, the various physiological traits associated with salinity tolerance have not been found combined in any extant genotype and all the known salt-tolerant cultivars have either one or few of these traits (cf. Yeo et al., 1990). Useful genetic variation is present for each character both in presence/absence as well as in the extent of expression. Thus, salt tolerance of rice might be improved beyond the present phenotypic range by use of physiological criteria to select independently for individual contributing traits, which may subsequently be pyramided. Combining physiological traits causative to overall enhanced salinity tolerance in rice is a feasible but long-term ambition (Yeo and Flowers, 1986; Yeo et al., 1990).

The use of physiological criteria in breeding is, however, restricted by some important constraints. Many individuals are needed to obtain a single assessment (see Section 36.5.3.3) making selection in early generations virtually impossible. Secondly, screening methods can logically be used to select parental lines but cannot generally be used to select individuals from a large number of plants as the case in early segregating populations. Thirdly, the destructive nature of many of the physiological assays renders them impractical for field screening especially in situations where the whole plant needs to be sampled. A final constraint is that in order to differentiate between stress-tolerant and susceptible phenotypes it is often necessary to expose plants to salt concentrations that are lethal to the sensitive individuals. Evaluation and selection may be feasible only in advanced generations. Developing strategies that use molecular markers to trace putative quantitative trait loci (QTL) presumed to underlie the physiological phenotype might surmount these problems.

### 36.10 EXPRESSION ANALYSIS TO IDENTIFY THE FACTORS RESPONSIBLE FOR SALT STRESS

Expression profiling was used for the first time in rice in response to abiotic stress by Kawasaki et al. (2001), who compared the gene expression profiles in salt-tolerant (var. Pokkali) and salt-sensitive (var. IR29) rice in response to salt stress. The analysis was performed using a cDNA microarray comprising 1728 cDNA clones prepared from unstressed or salt-stressed roots of Pokkali. The main difference between the expression patterns of the two varieties was the delayed response of IR29 for its defense gene expression, while Pokkali showed rapid expression of genes that could be responsible for the difference in tolerance (Kawasaki et al., 2001). Walia et al. (2005) used the Affymetrix rice genome array containing 55,515 probe sets to explore the transcriptome of salt-tolerant and salt-sensitive rice genotypes under control and salinity-stressed conditions during vegetative growth. They used two *indica* rice genotypes, FL478 (IR66948-3R-178-1-1-1, a recombinant inbred line derived from a cross between IR29 and Pokkali) and IR29, the sensitive parent of the population, for their study. A relatively large number of the probe sets were induced in the sensitive genotype in response of salt stress compared to tolerant FL478.

A genome-wide microarray has been used to monitor expression changes for a total of 36,926 genes in response to drought and high-salinity stresses in shoots at the four-tiller stage and in flag leaves and panicles at 1 week prior to heading from an *indica* cultivar Minghui 63 (Zhou et al., 2007). Plants were sampled at three different growth stages after growing in drought and salinity (200 mM). A large number of up-regulated and down-regulated genes were identified in flag leaf, shoot, and panicle, out of which many known genes responsive to both drought and high-salinity stress were recovered from the microarray analysis. These included the LEA protein (OsJRFA063984), aquaporin (OsIRUA001311), OsNAC1 (OsJRFA108080), dehydrin rab 16b (OsIFCC035025) and DREB1 (Os-JRFA067313). A major finding was the commonality of the genes for salinity and drought stress within each organ. A total of 322, 415, and 174 genes were up-regulated and 215, 173, and 372 genes were down-regulated by both stresses in flag leaves, shoots, and panicles, respectively. The common genes represent 55%, 33%, and 28% (induced) and 27%, 27%, and 29% (repressed) of all drought-responsive genes in flag leaf, shoot, and panicle, respectively. These results indicate that about one-third to a half (flag leaves) of the drought responsive genes in each organ are also regulated by high salinity stress (Zhou et al., 2007).

Many other genes have been reported up-regulated by salt—too many for us to discuss these here. However, it is worth mentioning that the gene (*OsNHX1*) for the  $\text{Na}^+/\text{H}^+$  antiporter (NHX), which is involved in sequestering  $\text{Na}^+$  within vacuoles has been cloned from rice and showed enhanced (two fold) expression in roots as well as shoots after 24 h of salt treatment (Fukuda et al., 1999, 2004). Another antiporter, with a significant similarity to a plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter “AtNHA1” from *Arabidopsis thaliana*, was up-regulated in both shoots and roots of rice seedlings under salt stress, whereas it was not induced in the rice seedlings treated by drought stress (Zhou et al., 2006).

### 36.11 TRANSGENICS

In spite of the complexity of salt tolerance, claims are common in the literature that the transfer of a single or a few genes can increase the high level of tolerance of plants to saline conditions. Transgenic plants are those that contain gene(s) that are artificially inserted from another unrelated organism and commonly known as genetically modified (GM) plants. The world's first transgenic plant was reported in 1983, and most of the transgenics that have been produced were made for herbicidal tolerance especially in soybean and maize. Recent reviews on transgenics in rice provide

a good overview of the transgenic research for herbicide, biotic, abiotic, and nutritional factors (Cherian et al., 2006; Kathuria et al., 2007). For salt stress, transgenics have been produced since 1993 (see Flowers, 2004) with the preponderance involving arabidopsis; about 45 papers, however, report transgenic rice with claims for altered salt tolerance.

A study conducted by Flowers (2004) showed that, of the 68 papers produced between 1993 and early 2003, only 19 reported quantitative estimates of plant growth. About half of all the papers showed data on experiments conducted under conditions where there was little or no transpiration: such experiments may provide insights into components of tolerance, but they cannot qualify for the claims of enhanced tolerance at the whole plant level. Appendix 36.B.1A lists nearly 40 papers in which rice has been transformed and where a claim is made in the title or the abstract of the paper that the transformant showed enhanced salt tolerance. The papers have been categorized according to the criteria developed by Flowers (2004) as it is important to distinguish between assessments of tolerance made *in vitro* (category 3) and those where experiments provide quantitative data on transformants and a control under saline and nonsaline conditions (category 1). Where there is little or no transpiration, experiments have less relevance to field situations than assessments made in greenhouses (or even fields) although such *in vitro* experiments do provide some information on the action of the gene product. It is apparent from the experiments classed as category 1 that increasing the synthesis of compatible solutes such as glycine betaine (Su et al., 2006) or proline does enhance growth (Anoop and Gupta, 2003) and yield (Wu et al., 2003) under saline conditions. Improvements in tolerance have also been seen in transgenic plants that express a  $\text{Na}^+/\text{H}^+$  antiporter from yeast (SOD2 Zhao et al., 2006a) and various transcription factors (SNAC1 Hu et al., 2006; ZFP252 Xu et al., 2008; ONAC045 Zheng et al., 2009) as does the production of sedoheptulose-1,7-bisphosphatase in chloroplasts (Feng et al., 2007). However, the expression of some genes can enhance sensitivity to salt (see Appendix 36.B.2B: aquaporin Katsuhara et al., 2003; nonexpressor of pathogenesis-related protein Quilis et al., 2008; and the transcription factor OsABI5 Zou et al., 2008). While some researchers continue to assess tolerance using germination or plantlets *in vitro*, what is really needed is field assessment, since after more than 10 years of research using transgenic rice to alter salt tolerance, the value of this approach has yet to be established in the field.

## 36.12 CONCLUSIONS

Salinity has affected agriculture over the millennia (Flowers and Yeo, 1995), but we might now expect its impact to be greater than in previous decades as climate change and population growth combine to present major challenges to world food production. Rice will remain one of the world's most important crops and, if the extent of waterlogging were to increase, it will assume even greater importance, since our other major cereals are not tolerant of flooding. Nevertheless, the sensitivity of rice to salinity is a drawback that needs to be overcome and past experience suggests that producing rice with salinity tolerance approaching that of barley will be difficult to achieve. However, our understanding of the complexity of salt tolerance has increased greatly since the early analyses of Akbar et al. (1972); we now know much more of the underlying traits (see Section 36.5) and of some of the genes involved. Furthermore, the existing variability in terms of the high level of tolerance in some land races has maintained optimism among researchers that they can improve the level of salt tolerance in an agronomically superior background. It is to be hoped that use of marker assisted breeding, based on QTL analyses (see Section 36.7), will aid progress since there are both savings in cost and time over conventional breeding (Alpuerto et al., 2009). However, there are still significant challenges as there are important gaps in our knowledge of, for example, the uptake and transport of  $\text{Na}^+$  and  $\text{Cl}^-$  in rice. Producing salt-tolerant rice will require ingenuity and the cooperation of scientists from across the world.

TABLE 36.A.1  
QTLs/Gene Identified for Salt Tolerance

| Gene/QTLs              | Chrom No. | Probable Position<br>(cM or Mb) of<br>QTL or Nearest<br>Marker | Trait                      | LOD   | Phenotypic<br>Variation<br>(%) | Flanking/Nearest<br>Marker | References                |
|------------------------|-----------|----------------------------------------------------------------|----------------------------|-------|--------------------------------|----------------------------|---------------------------|
| <i>salT</i>            | 1         | 13.8 Mb                                                        | Stress-induced protein     | —     | —                              | RG146B                     | Claes et al. (1990)       |
| Salt tolerance         | 7         | 7.0 ± 2.9 cM                                                   | Score-based salt tolerance | —     | —                              | RG4                        | Zhang et al. (1995)       |
| Trait-based QTL        | 7         | 8.1 ± 3.1 cM                                                   | 1000 grain weight          | —     | —                              | RG4                        | —                         |
| <i>Saltol</i>          | 1         | 14.7 cM                                                        | K absorption               | 17.23 | 80.2                           | P3/M9-8- <i>Saltol</i>     | Gregorio (1997,<br>118pp) |
| <i>Saltol</i>          | 1         | 14.7 cM                                                        | Na absorption              | 14.54 | 64.6                           | P3/M9-8- <i>Saltol</i>     | —                         |
| <i>Saltol</i>          | 1         | 14.7 cM                                                        | Na–K ratio                 | 14.51 | 64.3                           | P3/M9-8- <i>Saltol</i>     | —                         |
| Trait-based QTL        | 3         | 4.1 cM                                                         | Na absorption              | 3.17  | 17.1                           | P1/M5-3-P3/M9-1            | —                         |
| Trait-based QTL        | 3         | 0.6 cM                                                         | Na absorption              | 3.02  | 16                             | P1/M10-6-P1/<br>M7-10      | —                         |
| Trait-based QTL        | 4         | 0.0 cM                                                         | K absorption               | 5.34  | 83.5                           | RG375-P4/M3-2              | —                         |
| Trait-based QTL        | 10        | 32.3 cM                                                        | Na absorption              | 3.96  | 35.6                           | P1/M3-10-P1/<br>M3-8       | —                         |
| Trait-based QTL        | 10        | 65.6 cM                                                        | Na–K ratio                 | 3.6   | 86.1                           | G291-P1/M7-8               | —                         |
| Trait-based QTL        | 12        | 21.2 cM                                                        | K absorption               | 3.46  | 21.2                           | P1/M1-3-P2/M1-3            | —                         |
| Trait-based QTL        | 12        | 21.2 cM                                                        | Na–K ratio                 | 3.14  | 18.5                           | P1/M1-3-P2/M1-3            | —                         |
| <i>qSDM-5</i>          | 5         | —                                                              | Seedling dry matter        | 2.88  | 17.9                           | RZ70-RZ225                 | Prasad et al. (2000)      |
| <i>qSGEM-6</i>         | 6         | —                                                              | Seed germination           | 2.74  | 16.3                           | RZ398-RG213                | —                         |
| <i>qSRTL-6</i>         | 6         | —                                                              | Seedling root length       | 2.85  | 18.9                           | RG 162-RG 653              | —                         |
| <i>qSDM-6</i>          | 6         | —                                                              | Seedling dry matter        | 2.47  | 16.7                           | CDO544-Amy2A               | —                         |
| <i>qSV-6</i>           | 6         | —                                                              | Seedling vigor             | 2.4   | 15.8                           | CDO544-Amy2A               | —                         |
| <i>qSGEM-7</i>         | 7         | —                                                              | Seed germination           | 2.83  | 19.5                           | CDO59-RG477                | —                         |
| <i>qSDM-10</i>         | 10        | —                                                              | Seedling dry matter        | 2.00  | 13.5                           | RZ625-RZ500                | —                         |
| <i>Q<sub>Na</sub></i>  | 1         | —                                                              | high sodium uptake         | —     | —                              | —                          | Flowers et al. (2000)     |
| <i>Q<sub>NaK</sub></i> | 4         | —                                                              | Na:K discrimination        | —     | —                              | —                          | —                         |



|                 |    |                                    |                                       |     |       |                                                 |                       |
|-----------------|----|------------------------------------|---------------------------------------|-----|-------|-------------------------------------------------|-----------------------|
| Q <sub>K1</sub> | 6  | —                                  | Potassium uptake                      | —   | —     | —                                               | —                     |
| Q <sub>K2</sub> | 9  | —                                  | Potassium uptake                      | —   | —     | —                                               | —                     |
| Trait-based QTL | 8  | 6.3 cM                             | Salt tolerance at veg. and rep. stage | —   | —     | RM223                                           | Lang et al. (2001)    |
| Trait-based QTL | 1  | 33.0 cM                            | Shoot Na–K ratio                      | —   | 9.14  | C86-RM212                                       | Lang et al. (2001)    |
| Trait-based QTL | 2  | —                                  | Shoot Na–K ratio                      | —   | 5.25  | C747                                            | —                     |
| Trait-based QTL | 2  | 45.3 cM                            | Shoot Na concentration                | —   | 7.22  | RM240-RM213                                     | —                     |
| Trait-based QTL | 3  | 33.1 cM                            | Root weight                           | —   | 9.34  | R3156-C563                                      | —                     |
| Trait-based QTL | 7  | 37.7 cM                            | Shoot Na–K ratio                      | —   | 5.86  | RM214-R1789                                     | —                     |
| Trait-based QTL | 9  | 16.1 cM                            | Root weight                           | —   | 6.35  | C397-C1454                                      | —                     |
| Trait-based QTL | 11 | 34.5 cM                            | Shoot weight                          | —   | 14.38 | RM209-RM206                                     | —                     |
| Trait-based QTL | 12 | 12.3 cM                            | Shoot K concentration                 | —   | 17.45 | G24-R1684                                       | —                     |
| Trait-based QTL | 12 | 12.3 cM                            | Shoot Na–K ratio                      | —   | 8.81  | G24-R1684                                       | —                     |
| Trait-based QTL | 1  | 74 cM                              | Na <sup>+</sup> uptake                | —   | 8.9   | E12M55-3                                        | Koyama et al. (2001)  |
| Trait-based QTL | 1  | 56 cM                              | K <sup>+</sup> concentration          | —   | 10.6  | E12M37-1                                        | —                     |
| Trait-based QTL | 1  | 74 cM                              | Na:K ratio                            | —   | 9.1   | E12M57-1                                        | —                     |
| Trait-based QTL | 4  | 10 cM                              | K <sup>+</sup> uptake                 | 3   | 6.8   | E12M65-1                                        | —                     |
| Trait-based QTL | 4  | 90 cM                              | K <sup>+</sup> concentration          | —   | 8.8   | E15M53-2                                        | —                     |
| Trait-based QTL | 4  | 24 cM                              | Na <sup>+</sup> concentration         | —   | 6.7   | E12M73-1;<br>E12M75-5;<br>E15M50-5;<br>E12M79-1 | —                     |
| Trait-based QTL | 4  | 14 cM                              | Na:K ratio                            | —   | 9.6   | E12M65-1                                        | —                     |
| Trait-based QTL | 6  | 34 cM                              | Dry mass                              | —   | 9.7   | E12M55-2                                        | —                     |
| Trait-based QTL | 6  | 30 cM                              | K <sup>+</sup> uptake                 | —   | 7.6   | OSR19;<br>E12M80-2                              | —                     |
| Trait-based QTL | 6  | 106 cM                             | Na <sup>+</sup> concentration         | —   | 6.4   | E12M35-2                                        | —                     |
| Trait-based QTL | 9  | 96 cM                              | K <sup>+</sup> uptake                 | —   | 19.6  | E12M55-4                                        | —                     |
| <i>Saltol</i>   | 1  | Within<br>51.6–65.9<br>cM/13.87 Mb | Na <sup>+</sup> uptake                | 5.8 | 39.2  | RM140-C1733S                                    | Bonilla et al. (2002) |
| <i>Saltol</i>   | 1  | Within<br>51.6–65.9<br>cM/13.87 Mb | K <sup>+</sup> uptake                 | 6.8 | 43.9  | RM140-C1733S                                    | —                     |

(continued)

**TABLE 36.A.1 (continued)**  
**QTLs/Gene Identified for Salt Tolerance**

| Gene/QTLs                | Chrom No. | Probable Position<br>(cM or Mb) of<br>QTL or Nearest<br>Marker | Trait                                | LOD       | Phenotypic<br>Variation<br>(%) | Flanking/Nearest<br>Marker | References                |
|--------------------------|-----------|----------------------------------------------------------------|--------------------------------------|-----------|--------------------------------|----------------------------|---------------------------|
| <i>Saltol</i>            | 1         | Within<br>51.6–65.9<br>cM/13.87 Mb                             | Na/K ratio                           | 6.6       | 43.2                           | RM140-C1733S               | —                         |
| <i>Saltol</i>            | 1         | 60.6–71.2 cM                                                   | Ion uptake and Na/K ratio            | 5.02      | 0.44                           | RM 140                     | Niones (2004, 78pp)       |
| <i>Saltol</i>            | 1         | 60.6–71.2 cM                                                   | Ion uptake and Na/K ratio            | 3.34      | 0.44                           | CP03970-CP3224             | —                         |
| Trait-based QTL          | 2         | Long arm                                                       | Tiller number                        | 2.22      | 12                             | C747-R3393                 | Takehisa et al.<br>(2004) |
| Trait-based QTL          | 2         | Long arm                                                       | Tiller number                        | 2.43–4.26 | 12–23                          | C1408-C560                 | —                         |
| Trait-based QTL          | 3         | Long arm                                                       | Shoot length                         | 2.99–5.30 | 14–24                          | C944-C595                  | —                         |
| Trait-based QTL          | 3         | Long arm                                                       | Shoot length                         | 2.30–2.80 | 14–17                          | R250-C136                  | —                         |
| Trait-based QTL          | 7         | Short arm                                                      | Shoot length                         | 3.45–4.7  | 16–22                          | R2401-R1488                | —                         |
| Trait-based QTL          | 7         | Short arm                                                      | Shoot length                         | 4.45      | 23                             | C1057-R565                 | —                         |
| <i>qSDS-1</i>            | 1         | —                                                              | Seedling survival                    | 4.88      | 18                             | C813-C86                   | Lin et al. (2004)         |
| <i>qSKC-1</i>            | 1         | —                                                              | Shoot K <sup>+</sup> concentration   | 11.74     | 40.1                           | C1211-S2139                | —                         |
| <i>qRNTQ-1</i>           | 1         | —                                                              | Root Na <sup>+</sup> total quantity  | 3.25      | 12.4                           | C813-C86                   | —                         |
| <i>qRKC-4</i>            | 4         | —                                                              | Root K <sup>+</sup> concentration    | 4.28      | 21.6                           | C891-C513                  | —                         |
| <i>qSDS-6</i>            | 6         | —                                                              | Seedling survival                    | 3.63      | 17                             | C214-R2549                 | —                         |
| <i>qSDS-7</i>            | 7         | —                                                              | Seedling survival                    | 3.32      | 13.9                           | R2401-L538T7               | —                         |
| <i>qSNC-7</i>            | 7         | —                                                              | Shoot Na <sup>+</sup> concentration  | 7.66      | 48.5                           | C1057-R2401                | —                         |
| <i>qSNTQ-7</i>           | 7         | —                                                              | Shoot Na <sup>+</sup> total quantity | 4.26      | 16.1                           | C1057-R2401                | —                         |
| <i>qRKC-7</i>            | 7         | —                                                              | Root K <sup>+</sup> concentration    | 3.48      | 17.8                           | C1057-R2401                | —                         |
| <i>qRKTQ-7</i>           | 7         | —                                                              | Root K <sup>+</sup> total quantity   | 3.82      | 17.3                           | C1057-R2401                | —                         |
| <i>qRNC-9</i>            | 9         | —                                                              | Root Na <sup>+</sup> concentration   | 3.25      | 16.7                           | R1751-R2638                | —                         |
| <i>SKC-1<sup>a</sup></i> | 1         | 11.46 Mb                                                       | Shoot K <sup>+</sup> concentration   | 10        | —                              | CAPS markers<br>K159-K061  | Ren et al. (2005)         |
| <i>qSTR-1</i>            | 1         | —                                                              | Salinity tolerance rating            | 2.06      | 6.7                            | RM9-RM128                  | Yao et al. (2005)         |
| <i>qSTR-5</i>            | 5         | —                                                              | Salinity tolerance rating            | 2.72      | 14.3                           | RM161-RM13                 | —                         |

|                 |    |           |                                                |       |       |                 |                           |
|-----------------|----|-----------|------------------------------------------------|-------|-------|-----------------|---------------------------|
| <i>qSTR-9</i>   | 9  | —         | Salinity tolerance rating                      | 2.06  | 7     | RM278-RM215     | —                         |
| <i>qNAK-2</i>   | 2  | —         | Na <sup>+</sup> /K <sup>+</sup> ratio in roots | 2.73  | 19.3  | RM318-RM262     | —                         |
| <i>qNAK6</i>    | 6  | —         | Na <sup>+</sup> /K <sup>+</sup> ratio in roots | 2.03  | 7.9   | RM176-RM345     | —                         |
| <i>qDWS-8</i>   | 8  | —         | Dry matter weight of shoot                     | 2.47  | 7.5   | RM223-RM152     | —                         |
| <i>qDWS-9</i>   | 9  | —         | Dry matter weight of shoot                     | 2.31  | 11.5  | RM278-RM215     | —                         |
| <i>qST1</i>     | 1  | —         | Seedling tolerance                             | 11.58 | 27.76 | Est1-2 & RZ569A | Lee et al. (2006a,b)      |
| <i>qST3</i>     | 3  | —         | Seedling tolerance                             | 3.39  | 9.16  | RG179-RZ596     | —                         |
| <i>qLB-3</i>    | 3  | Long arm  | Leaf bronzing                                  | 31.7  | 83    | R1925           | Takehisa et al.<br>(2006) |
| <i>qLB-11</i>   | 11 | Short arm | Leaf bronzing                                  | 3.5   | 8     | C1350-C477      | —                         |
| Seedling stage  |    |           |                                                |       |       |                 |                           |
| <i>QSst2</i>    | 2  | RM250     | Score of salt toxicity of leaves               | —     | —     | RM250-RM208     | Zang et al. (2008)        |
| <i>QSst3</i>    | 3  | RM231     | Score of salt toxicity of leaves               | —     | —     | RM231-RM175     |                           |
| <i>QSst8</i>    | 8  | RM38      | Score of salt toxicity of leaves               | —     | —     | RM38-RM25       |                           |
| <i>QSds2a</i>   | 2  | OSR17     | Survival days of seedlings                     | —     | —     | OSR17-RM211     |                           |
| <i>QSds2b</i>   | 2  | RM250     | Survival days of seedlings                     | —     | —     | RM530-RM250     |                           |
| <i>QSds3</i>    | 3  | RM231     | Survival days of seedlings                     | —     | —     | RM231-RM175     |                           |
| <i>QSds8</i>    | 8  | RM38      | Survival days of seedlings                     | —     | —     | RM38-RM25       |                           |
| <i>QSkc1</i>    | 1  | RM562     | Shoot K <sup>+</sup> concentration             | —     | —     | RM562-RM9       |                           |
| <i>QSkc3</i>    | 3  | RM81B     | Shoot K <sup>+</sup> concentration             | —     | —     | RM81B-RM22      |                           |
| <i>QSkc6</i>    | 6  | RM539     | Shoot K <sup>+</sup> concentration             | —     | —     | RM50-RM539      |                           |
| <i>QSkc11</i>   | 11 | RM120     | Shoot K <sup>+</sup> concentration             | —     | —     | RM120-RM181     |                           |
| <i>QSnc3</i>    | 3  | RM175     | Shoot Na <sup>+</sup> concentration            | —     | —     | RM231-RM175     |                           |
| <i>QSnc6</i>    | 6  | RM3       | Shoot Na <sup>+</sup> concentration            | —     | —     | RM527-RM3       |                           |
| Tillering stage |    |           |                                                |       |       |                 |                           |
| <i>QPh1</i>     | 1  | RM243     | Plant height                                   | —     | —     | RM243-RM600     |                           |
| <i>QPh4</i>     | 4  | RM142     | Plant height                                   | —     | —     | RM142-RM273     |                           |
| <i>QPh5</i>     | 5  | RM122     | Plant height                                   | —     | —     | RM122-RM13      |                           |
| <i>QPh7</i>     | 7  | RM248     | Plant height                                   | —     | —     | RM172-RM248     |                           |
| <i>QPh8</i>     | 8  | RM210     | Plant height                                   | —     | —     | RM223-RM210     |                           |
| <i>QPh9</i>     | 9  | RM409     | Plant height                                   | —     | —     | RM105-RM409     |                           |
| <i>QTn1</i>     | 1  | RM562     | Tiller number                                  | —     | —     | RM562-RM9       |                           |

(continued)

**TABLE 36.A.1 (continued)**  
**QTLs/Gene Identified for Salt Tolerance**

| Gene/QTLs      | Chrom No. | Probable Position<br>(cM or Mb) of<br>QTL or Nearest<br>Marker | Trait               | LOD  | Phenotypic<br>Variation<br>(%) | Flanking/Nearest<br>Marker | References                    |
|----------------|-----------|----------------------------------------------------------------|---------------------|------|--------------------------------|----------------------------|-------------------------------|
| <i>QTn2</i>    | 2         | RM71                                                           | Tiller number       | —    | —                              | RM71-RM324                 | Sabouri and Sabouri<br>(2008) |
| <i>QTn5</i>    | 5         | RM122                                                          | Tiller number       | —    | —                              | RM122-RM13                 |                               |
| <i>QTn6</i>    | 6         | RM30                                                           | Tiller number       | —    | —                              | RM528-RM30                 |                               |
| <i>QTn8</i>    | 8         | RM42                                                           | Tiller number       | —    | —                              | RM339-RM42                 |                               |
| <i>QTn9</i>    | 9         | RM566                                                          | Tiller number       | —    | —                              | RM409-RM566                |                               |
| <i>QTn10</i>   | 10        | RM239                                                          | Tiller number       | —    | —                              | RM222-RM239                |                               |
| <i>QFw1</i>    | 1         | RM575                                                          | Fresh weight        | —    | —                              | RM575-RM259                |                               |
| <i>QFw2a</i>   | 2         | RM71                                                           | Fresh weight        | —    | —                              | RM71-RM324                 |                               |
| <i>QFw2b</i>   | 2         | RM318                                                          | Fresh weight        | —    | —                              | RM318 - RM530              |                               |
| <i>QFw4</i>    | 4         | RM335                                                          | Fresh weight        | —    | —                              | RM335-RM261                |                               |
| <i>QFw7</i>    | 7         | RM10                                                           | Fresh weight        | —    | —                              | RM10-RM234                 |                               |
| <i>QFw8</i>    | 8         | RM25                                                           | Fresh weight        | —    | —                              | RM38-RM25                  |                               |
| <i>QFw10</i>   | 10        | RM467                                                          | Fresh weight        | —    | —                              | RM239-RM467                |                               |
| <i>QFw11a</i>  | 11        | RM181                                                          | Fresh weight        | —    | —                              | RM181-RM260                |                               |
| <i>QFw11b</i>  | 11        | RM21                                                           | Fresh weight        | —    | —                              | RM229-RM21                 |                               |
| <i>qPL-2</i>   | 2         | —                                                              | Plant stand         | 3.15 | 16.45                          | RM5699-RM262               |                               |
| <i>qCHLC-3</i> | 3         | —                                                              | Chlorophyll content | 4.67 | 14.5                           | RM1022-RM6283              |                               |
| <i>qRL-1</i>   | 1         | —                                                              | Root length         | 2.70 | 13.63                          | RM8068-RM8231              |                               |
| <i>qRL-4</i>   | 4         | —                                                              | Root length         | 2.91 | 11.56                          | RM5473-RM551               |                               |
| <i>qRL-5</i>   | 5         | —                                                              | Root length         | 3.67 | 14.8                           | RM421-RM480                |                               |
| <i>qRL-7</i>   | 7         | —                                                              | Root length         | 4.24 | 16.21                          | RM1048-RM11                |                               |
| <i>qRL-9a</i>  | 9         | —                                                              | Root length         | 4.47 | 14.12                          | RM1553-RM5702              |                               |
| <i>qRL-9b</i>  | 9         | —                                                              | Root length         | 3.58 | 11.51                          | RM7424-RM5702              |                               |
| <i>qSHL-3</i>  | 3         | —                                                              | Shoot length        | 2.69 | 23.57                          | RM7389-RM7000              |                               |
| <i>qSHL-10</i> | 10        | —                                                              | Shoot length        | 5.30 | 19.19                          | RM7545-RM4455              |                               |
| <i>qGLA-3</i>  | 3         | —                                                              | Green leaf area     | 5.54 | 12.81                          | RM1022-RM6283              |                               |

|                 |    |   |                                         |       |       |               |
|-----------------|----|---|-----------------------------------------|-------|-------|---------------|
| <i>qFWSH-1</i>  | 1  | — | Fresh weight shoot                      | 3.36  | 22.44 | RM8235-RM8144 |
| <i>qFWSH-3</i>  | 3  | — | Fresh weight shoot                      | 5.01  | 22.97 | RM1022-RM6283 |
| <i>qFWRO-3a</i> | 3  | — | Fresh weight root                       | 4.70  | 20.91 | RM1022-RM6283 |
| <i>qFWRO-3b</i> | 3  | — | Fresh weight root                       | 4.64  | 17.72 | RM6283-RM6832 |
| <i>qDWSH-3</i>  | 3  | — | Dry weight shoot                        | 4.46  | 23.21 | RM1022-RM6283 |
| <i>qDWSH-7</i>  | 7  | — | Dry weight shoot                        | 2.84  | 23.17 | RM5481-RM11   |
| <i>qDWRO-3</i>  | 3  | — | Dry weight root                         | 3.58  | 21.41 | RM1022-RM6283 |
| <i>qDWRO-5a</i> | 5  | — | Dry weight root                         | 2.65  | 21.74 | RM421-RM480   |
| <i>qDWRO-5b</i> | 5  | — | Dry weight root                         | 2.87  | 22.55 | RM480-RM440   |
| <i>qDWRO-9a</i> | 9  | — | Dry weight root                         | 3.24  | 27.43 | RM1553-RM7424 |
| <i>qDWRO-9b</i> | 9  | — | Dry weight root                         | 2.95  | 25.5  | RM7424-RM5702 |
| <i>qNAUP-1a</i> | 1  | — | Na <sup>+</sup> uptake                  | 2.84  | 13.03 | RM562-RM543   |
| <i>qNAUP-1b</i> | 1  | — | Na <sup>+</sup> uptake                  | 5.60  | 22.17 | RM8068-RM8231 |
| <i>qNAUP-3</i>  | 3  | — | Na <sup>+</sup> uptake                  | 2.81  | 13.62 | RM416-RM5626  |
| <i>qNAUP-9a</i> | 9  | — | Na <sup>+</sup> uptake                  | 4.97  | 17.71 | RM1553-RM7424 |
| <i>qNAUP-9b</i> | 9  | — | Na <sup>+</sup> uptake                  | 4.43  | 16.95 | RM7424-RM5702 |
| <i>qNAUP-10</i> | 10 | — | Na <sup>+</sup> uptake                  | 3.80  | 13.84 | RM7545-RM4455 |
| <i>qKUP-3</i>   | 3  | — | K <sup>+</sup> uptake                   | 4.48  | 22.15 | RM1022-RM6283 |
| <i>qKUP-8</i>   | 8  | — | K <sup>+</sup> uptake                   | 5.16  | 38.22 | RM4955-RM152  |
| <i>qNAKUP-6</i> | 6  | — | Na <sup>+</sup> /K <sup>+</sup> ratio   | 3.83  | 12.35 | RM3827-RM340  |
| <i>qNAKUP-3</i> | 3  | — | Na <sup>+</sup> /K <sup>+</sup> ratio   | 4.25  | 9.03  | RM6832-RM7389 |
| <i>qSTR-6</i>   | 6  | — | Standard tolerance ranking <sup>b</sup> | 17.51 | 17.25 | RM3727-RM340  |
| <i>qSTR-3a</i>  | 3  | — | Standard tolerance ranking <sup>b</sup> | 13.44 | 16.15 | RM1022-RM6283 |
| <i>qSTR-3b</i>  | 3  | — | Standard tolerance ranking <sup>b</sup> | 24.51 | 13.07 | RM6832-RM7389 |
| <i>qDM-3</i>    | 3  | — | Dry mass of shoot                       | 20.5  | 20.9  | RM1022-RM6283 |
| <i>qDM-8</i>    | 8  | — | Dry mass of shoot                       | 20.24 | 17.72 | RM4955-RM152  |
| <i>qNA-2a</i>   | 2  | — | Na <sup>+</sup> content in shoot        | 12.59 | 10.55 | RM8264-RM262  |
| <i>qNA-2b</i>   | 2  | — | Na <sup>+</sup> content in shoot        | 12.57 | 12.7  | RM7426-RM236  |
| <i>qNA-6</i>    | 6  | — | Na <sup>+</sup> content in shoot        | 11.92 | 10.13 | RM3827-RM5371 |
| <i>qNA-3</i>    | 3  | — | Na <sup>+</sup> content in shoot        | 16.34 | 10.92 | RM6832-RM7389 |
| <i>qK-6</i>     | 6  | — | K <sup>+</sup> content in shoot         | 14.46 | 10.8  | RM3827-RM340  |
| <i>qK-5a</i>    | 5  | — | K <sup>+</sup> content in shoot         | 17.16 | 15.58 | RM421-RM480   |
| <i>qK-5b</i>    | 5  | — | K <sup>+</sup> content in shoot         | 12.35 | 9.7   | RM480-RM440   |

Sabouri et al. (2009)

(continued)

**TABLE 36.A.1 (continued)**  
**QTLs/Gene Identified for Salt Tolerance**

| Gene/QTLs                                   | Chrom No. | Probable Position<br>(cM or Mb) of<br>QTL or Nearest<br>Marker | Trait                                                               | LOD   | Phenotypic<br>Variation<br>(%) | Flanking/Nearest<br>Marker | References          |
|---------------------------------------------|-----------|----------------------------------------------------------------|---------------------------------------------------------------------|-------|--------------------------------|----------------------------|---------------------|
| <i>qNAK-6</i>                               | 6         | —                                                              | Na <sup>+</sup> /K <sup>+</sup> ratio                               | 16.68 | 12.35                          | RM3827-RM340               | Ammar et al. (2009) |
| <i>qNAK-3</i>                               | 3         | —                                                              | Na <sup>+</sup> /K <sup>+</sup> ratio                               | 18.52 | 9.03                           | RM6832-RM7389              |                     |
| <i>qCl-SV-1.1</i>                           | 1         | 86.4 cM                                                        | Cl <sup>-</sup> in stem at vegetative stage                         | 6.45  | 55.72                          | RM572-RM294                |                     |
| <i>qNa<sup>+</sup> SV-1.1</i>               | 1         | 84.4 cM                                                        | Na <sup>+</sup> in stem at vegetative stage                         | 6.62  | 52.27                          | RM572-RM294                |                     |
| <i>qCl-SV-2.1</i>                           | 2         | 73.8 cM                                                        | Cl <sup>-</sup> in stem at vegetative stage                         | 5.89  | 42.5                           | RM145-RM5699               |                     |
| <i>qCl-LR-2.1</i>                           | 2         | 69.8 cM                                                        | Cl <sup>-</sup> in leaf at reproductive stage                       | 7.57  | 26.26                          | RM145-RM5699               |                     |
| <i>qNa<sup>+</sup> SV-2.1</i>               | 2         | 77.8 cM                                                        | Na <sup>+</sup> in stem at vegetative stage                         | 6.57  | 49                             | RM145-RM5699               |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>SV-2.1</i>  | 2         | 52.8 cM                                                        | Na <sup>+</sup> /K <sup>+</sup> ratio in stem at vegetative stage   | 8.18  | 46.57                          | RM3732-RM145               |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>SV-2.2</i>  | 2         | 56.8 cM                                                        | Na <sup>+</sup> /K <sup>+</sup> ratio in stem at vegetative stage   | 10.08 | 45.5                           | RM3732-RM145               |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>SV-2.3</i>  | 2         | 73.8 cM                                                        | Na <sup>+</sup> /K <sup>+</sup> ratio in stem at vegetative stage   | 9.7   | 42.88                          | RM145-RM5699               |                     |
| <i>qNa<sup>+</sup>LV-3.1</i>                | 3         | 160.8 cM                                                       | Na <sup>+</sup> in leaves at vegetative stage                       | 6.6   | 53.8                           | RM563-RM186                |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>LR-3.1</i>  | 3         | 160.8 cM                                                       | Na <sup>+</sup> /K <sup>+</sup> ratio in leaf at reproductive stage | 9.32  | 52.63                          | RM563-RM186                |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>SV-3.1</i>  | 3         | 164.8 cM                                                       | Na <sup>+</sup> /K <sup>+</sup> ratio in stem at vegetative stage   | 5.29  | 47.51                          | RM563-RM186                |                     |
| <i>qCl-LV-3.1</i>                           | 3         | 158.8 cM                                                       | Cl <sup>-</sup> in leaf at vegetative stage                         | 7.78  | 48.51                          | RM563-RM186                |                     |
| <i>qNa<sup>+</sup>LV-8.2</i>                | 8         | 130.5 cM                                                       | Na <sup>+</sup> in leaves at vegetative stage                       | 4.78  | 55.18                          | RM3395-RM281               |                     |
| <i>qNa<sup>+</sup>LR-8.1</i>                | 8         | 122.5 cM                                                       | Na <sup>+</sup> in leaf at reproductive stage                       | 3.95  | 47.59                          | RM3395-RM281               |                     |
| <i>qNa<sup>+</sup>SV-8.1</i>                | 8         | 124.5 cM                                                       | Na <sup>+</sup> in stem at vegetative stage                         | 4.72  | 53.63                          | RM3395-RM281               |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup>LR-8.1</i>  | 8         | 126.5 cM                                                       | Na <sup>+</sup> /K <sup>+</sup> ratio in leaf at vegetative stage   | 6     | 51.03                          | RM3395-RM281               |                     |
| <i>qNa<sup>+</sup>/K<sup>+</sup> SV-8.1</i> | 8         | 126.5 cM                                                       | Na <sup>+</sup> /K <sup>+</sup> ratio in stem at vegetative stage   | 4.84  | 51.78                          | RM3395-RM281               |                     |

<sup>a</sup> Gene mapped by map-based cloning.

<sup>b</sup> STR is same as SES (IRRI).

## APPENDIX 36.B

TABLE 36.B.1

**A Chronological List of Transformations of Rice with Claims for Enhanced Tolerance to (A) or Sensitivity to (B) Salinity**

| Data         | Plant Material              | Treatments              | Category |
|--------------|-----------------------------|-------------------------|----------|
| Quantitative | Transformants and wild type | Plus and minus salinity | 1        |
| Quantitative | Material missing            | Treatments missing      | 1a       |
| Qualitative  | Transformants and wild type | Plus and minus salinity | 2        |
| Qualitative  | Material missing            | Treatments missing      | 2a       |
| All in vitro | —                           | —                       | 3        |

| Transform                          | Product                                     | Class of Product             | Category | Citation                |
|------------------------------------|---------------------------------------------|------------------------------|----------|-------------------------|
| <b>A</b>                           |                                             |                              |          |                         |
| <i>HVA1</i>                        | LEA                                         | Protective protein           | 1a       | Xu et al. (1996)        |
| <i>badH</i>                        | Glycinebetaine                              | Compatible solute            | 2a       | Guo et al. (1997)       |
| <i>Choline oxidase</i>             | Glycinebetaine                              | Compatible solute            | 2a       | Sakamoto et al. (1998)  |
| <i>P5CS</i>                        | Proline                                     | Compatible solute            | 3        | Zhu et al. (1998)       |
| <i>Glutamine synthetase</i>        | Glutamine synthetase                        | Photo-respiratory metabolism | 3        | Hoshida et al. (2000)   |
| <i>badH</i>                        | Glycinebetaine                              | Compatible solute            | 3        | Kishitani et al. (2000) |
| <i>OsCDPK7</i>                     | Protein kinase                              | Signaling                    | 3        | Saijo et al. (2000)     |
| <i>mtlD</i>                        | Mannitol                                    | Compatible solute            | 2a       | Wang et al. (2000)      |
| <i>ADC</i>                         | Arginine decarboxylase                      | Miscellaneous                | 3        | Roy and Wu (2001)       |
| <i>CDPK</i>                        | Protein kinase                              | Signaling                    | 3        | Saijo et al. (2001)     |
| <i>LEA</i>                         | LEA protein                                 | Protective protein           | 1a       | Cheng et al. (2002)     |
| <i>TPSP</i>                        | Trehalose                                   | Compatible solute            | 1a       | Garg et al. (2002)      |
| <i>RHL HAL2-like gene</i>          | Bisphosphate-3'-nucleotidase?               | Signaling                    | 3        | Li et al. (2002)        |
| <i>codA</i>                        | Glycinebetaine                              | Compatible solute            | 3        | Mohanty et al. (2002)   |
| <i>NHX1</i>                        | Na H <sup>+</sup> antiporter                | Transport related protein    | 2a       | Ohta et al. (2002)      |
| <i>HVA1</i>                        | LEA protein                                 | Protective protein           | 1a       | Rohila et al. (2002)    |
| <i>SAMDC cDNA</i>                  | S-Adenosylmethionine decarboxylase          | Miscellaneous                | 3        | Roy et al. (2002)       |
| <i>P5CS</i>                        | Proline                                     | Compatible solute            | 1a       | Anoop and Gupta (2003)  |
| <i>d-OAT</i>                       | Δ-Pyrroline-5-carboxylate                   | Compatible solute            | 1        | Wu et al. (2003)        |
| <i>OsMAPK5</i>                     | Mitogen activated protein kinase            | Signaling                    | 2        | Xiong et al. (2003)     |
| <i>NHX1</i>                        | Na H <sup>+</sup> antiporter                | Transport related protein    | 2        | Fukuda et al. (2004)    |
| <i>PvMet1</i>                      | Metallothionein and UDP-galactose epimerase | Miscellaneous                | 2        | Endo et al. (2005)      |
| <i>CBF3/DREB1A (CBF3) and ABF3</i> | Transcription factors                       | Transcription factor         | 3        | Oh et al. (2005)        |

(continued)

**TABLE 36.B.1 (continued)**  
**A Chronological List of Transformations of Rice with Claims for Enhanced Tolerance to (A) or Sensitivity to (B) Salinity**

| Transform                                                      | Product                                             | Class of Product             | Category | Citation                |
|----------------------------------------------------------------|-----------------------------------------------------|------------------------------|----------|-------------------------|
| <i>SNAC1</i>                                                   | Transcription factor                                | Transcription factor         | 1        | Hu et al. (2006)        |
| <i>Choline monooxygenase</i>                                   | Choline monooxygenase                               | Compatible solute            | 2        | Shirasawa et al. (2006) |
| <i>COX</i>                                                     | Glycinebetaine                                      | Compatible solute            | 1        | Su et al. (2006)        |
| <i>SOD2</i>                                                    | SOD2                                                | Transport related protein    | 1        | Zhao et al. (2006a)     |
| <i>SsNHX1</i>                                                  | Na/H antiporter                                     | Transport related protein    | 2        | Zhao et al. (2006b)     |
| <i>GST; CAT1</i>                                               | Glutathione <i>S</i> -transferase; catalase         | ROS related                  | 2        | Zhao and Zeng (2006a)   |
| <i>SsNHX1; AVP1</i>                                            | Na/H antiporter; Ppiase                             | Transport related protein    | 2        | Zhao et al. (2006c)     |
| Na/H antiporter                                                | Vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter | Transport related protein    | 2        | Chen et al. (2007)      |
| <i>Sedoheptulose-1,7-bisphosphatase</i>                        | SBPase in chloroplasts                              | Photo-respiratory metabolism | 1        | Feng et al. (2007)      |
| <i>OsCOIN</i>                                                  | bZIP zinc finger protein                            | Zinc finger protein          | 3        | Liu et al. (2007)       |
| <i>HvCBF4 CBF3/ DREB1A</i>                                     | HvCBF4 CBF3/ DREB1A                                 | Transcription factor         | 2a       | Oh et al. (2007)        |
| <i>PgNHX1</i>                                                  | Na/H antiporter                                     | Transport related protein    | 2        | Verma et al. (2007)     |
| Calcineurin B-like protein-interacting protein kinases (CIPKs) | Protein kinase                                      | Transcription factor         | 3        | Xiang et al. (2007)     |
| <i>OPBP1</i>                                                   | OPBP1                                               | Transcription factor         | 3        | Chen et al. (2008)      |
| <i>ZFP252</i>                                                  | TFIIIA-type zinc finger protein                     | Transcription factor         | 1        | Xu et al. (2008)        |
| <i>ONAC045</i>                                                 | NAC protein                                         | Transcription factor         | 1        | Zheng et al. (2009)     |
| <b>B</b>                                                       |                                                     |                              |          |                         |
| <i>SOD</i>                                                     | Superoxide dismutase                                | ROS related                  | 3        | Tanaka et al. (1999)    |
| <i>HvPIP2;1</i>                                                | Aquaporin                                           | Transport related protein    | 1        | Katsuhara et al. (2003) |
| <i>GST</i>                                                     | Glutathione <i>S</i> -transferase                   | ROS related                  | 3        | Zhao and Zeng (2006b)   |
| <i>AtNPR1</i>                                                  | Nonexpressor of pathogenesis-related (PR) protein   | Protective protein           | 1        | Quilis et al. (2008)    |
| <i>OsABI5</i>                                                  | OsABI5 protein                                      | Transcription factor         | 1        | Zou et al. (2008)       |

*Note:* The table lists the gene transferred, the product and a generic description of that product. The reports have also been categorized according to the following criteria.



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# 37 Landscape under Water-Stress Conditions

*Atif Riaz*

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### 37.1 INTRODUCTION

A modern landscape, in essence, is the outcome of human efforts to improve the aesthetic value of a given piece of land, which may vary in size from a small lawn in a house or land available around the public and private buildings to large public parks, playgrounds, golf courses, open spaces in a city, banks of canals or a river passing through a city, land along roadsides and railway tracks, etc. In the rural scenes, the whole land around the villages and towns could come under the scope of landscaping.

Generally speaking, it is the open area available in public and private properties, public parks, playgrounds, and open spaces in the city that gets the maximum attention of landscape developers. Such areas have ensured supply of freshwater through trickle, sprinkle, or even flow irrigation for sustained growth of a variety of plants occupying the area. But freshwater is a fragile resource. Korzun [1] emphasized that about 96.5% of this planet's water is highly saline seawater (about 35,000 ppm). Of the remainder, about 1.8% is frozen in icecaps, glaciers, etc., and 0.8% is in inaccessible underground aquifers. Thus, agriculture and human civilization are dependent upon less than 1% of the planet's water [2]. According to FAO estimates of 2007, approximately 1.2 billion people live in water-scarce countries [3]. This number is expected to increase with the increase in population and change in climate.

It has been experienced that, as the society confronts progression in water scarcity, the water taps for activities like car washing, road washing, and landscaping are turned off immediately to ensure continued supply of water for drinking, household use, industry, and agriculture. As such, moisture deficiency is the most common stress encountered in landscapes. Drought conditions for short periods may not seriously harm plants, but, if these persist for several months, they can seriously hamper plant growth and even kill plants. Effects of water deficit are reflected in reduced growth and agronomic yields of crops and plants through various morphological and physiological mechanisms. Passioura [4] developed the following relationship between yield and transpiration:  $Y = T \times WUE \times HI$ , where [Y] stands for yield, which is dependent on the volume of water transpired by the plant [T], the water use efficiency (WUE) of the system, and the harvest index [HI], i.e., the proportion of the total biomass produced that may be exploited by a particular farming system. In the case of ornamental plants, the look and the beauty of plants as determined by the size, number, color, luster, and shape of the leaves, flowers, and fruits is a relevant factor of more concern to landscape developers, as wilting and discoloration of lawns and foliage and fruit drop are commonly observed under moisture stress. In addition, these symptoms lead to additional complications, as various insect pests and fungal diseases find an easy prey in plants under stress [5].

For selecting the appropriate species for a given set of soil, water, and environmental conditions, not only the survival rate and the water-deficit threshold at which growth reduction takes place are important considerations, but also the relative rate of reduction in the above parameters with an increase in water deficit is an important character of the species.

The selection of appropriate plants for a particular landscape scenario depends on not only the level of water deficits, but also on many other factors, such as temperature; expected rate of evapotranspiration at different growth stages of the plant; atmospheric humidity; seasonal fluctuations in rainfalls and periodic droughts; soil factors, such as soil depth, texture, structure, organic matter contents, and water-holding capacity; as well as availability of alternative sources of irrigation, such as domestic effluents, sewage water, saline water, etc. Therefore, the selection of suitable plant types for landscape purposes under water-deficit conditions cannot be undertaken in isolation without considering the "on-site" factors enumerated above. It needs to be emphasized that water is only one of the many factors affecting the growth of ornamental plants and, in fact, many "non-water" factors may actually be having more impact on the growth of plants than water itself. This fact has nicely been elaborated by Wyn Jones and associates [2], who quoted data from ICARDA, which demonstrated that wheat yields of 4–5 t/ha could be achieved with the use of 300–400 mm of water under rainfed conditions in megarenvironments of SE Australia, China Loess Plateau, Mediterranean

Basin, and N. American Great Plains, compared with countries like Pakistan, having a yield of 1–2t/ha under similar conditions. Also, it is important that landscape practitioners in water-scarce areas are made aware of (1) the need for selecting the right kind of plant species, (2) alternatives for landscape water conversation, and (3) the use of alternative water resources. Finally, capacity building of the landscape practitioners in the science and art of sustainable management of landscapes under different water scarcity scenarios is necessary.

### 37.2 HOW DOES DROUGHT STRESS AFFECT PLANTS?

Moisture stress from drought affects landscape plants like any other plant species. The impact of drought on plant growth varies with the severity and duration of the drought combined with the other factors including plant species, stage of development [6], and soil conditions, whereas root injury to plants accentuates these effects.

Water enters the plant roots due to the gradient between the water potential outside and inside the root. The reduction in water potential due to loss of water or due to accumulation of salts at the soil–root interface reduces the rate of water entry into the root due to reduction in this gradient. Water stress affects plant growth via biophysical and physiological processes. No growth will take place if the turgor pressure of cells is below the threshold in relation to the cell wall extensibility, as given below:

$$GR = m(\Psi_p - \gamma)$$

where

GR is the growth rate

$\Psi_p$  is the turgor

$\gamma$  is the yield threshold (the pressure below which the cell wall resists plastic, or nonreversible, deformation)

$m$  is the wall extensibility (the responsiveness of the wall to pressure) [7]

Moreover, the growth is seriously hampered by stomatal closure under water stress as would be the nutrient uptake, cell wall synthesis, protochlorophyll formation,  $\text{CO}_2$  assimilation, and xylem conduction [8,9].

Although the general effects of drought on plant growth are quite well known, the primary effects of water deficit at the biochemical and molecular levels are not well understood [10,11]. Despite the lack of understanding of drought-tolerance mechanisms, physiological and molecular biological studies have documented several plant responses to drought stress [9,12–17]. Plants respond to drought stress in different ways; the first response to drought is the closure of leaf stomata [18], which reduces the carbon dioxide absorption due to which photosynthesis is curtailed [19]. This reduction in photosynthesis limits growth and increases susceptibility to insects/pests and diseases. In addition to less carbohydrate (food) production, protein contents, lipids, enzymes, growth regulators [20], mineral nutrients [21], and other essential materials for life are not produced and/or translocated, enzymatic activities are inhibited [22], and carbon and nitrogen metabolism is altered in the plant [23–28], resulting in reduced plant growth and development. Plant physiologists often classify plants into  $\text{C}_3$ ,  $\text{C}_4$ , or Crassulacean Acid Metabolism (CAM) groups that vary in their ability to fix  $\text{CO}_2$  under water stress conditions. Many CAM plants have a good landscape value as well.

Growth reduction is often most severe in the year following drought. Restricted growth and vitality are manifested by stunted chlorotic leaves, premature defoliation, interveinal necrosis, leaf scorch (Plate 37.1), unabscised dead leaves on the tree [29], crown thinning, and poor shoot growth. In turfgrass, water stress is typically characterized by the browning of leaves [30]. Landscape plants typically require several years to recover fully from drought due to a lesser capacity for food production.



**PLATE 37.1** Leaf scorch in *Syngonium podophyllum*.

### 37.2.1 EFFECTS OF ENVIRONMENT ON DROUGHT STRESS

Aside from the moisture content available in the soil, environmental conditions of high temperature, low relative humidity, high light intensity, and high wind speed increase plant water loss significantly through transpiration and evaporation. The prior environment of a plant also can influence the development of drought stress. A plant that has recovered after it has been drought-stressed previously may become more drought resistant. Also, a plant that was well watered prior to drought will usually survive the drought period better than a continuously drought-stressed plant [31].

### 37.2.2 RELATIONSHIP BETWEEN ROOT VOLUME AND DROUGHT TOLERANCE

A limited root system may also accelerate the rate at which drought stress affects plants. Root volume may be limited by the presence of a competing root system, or by site conditions such as waterlogging, salinity, compaction, or soil volume limited by the container (if growing in a container). A plant having a large size or more number of leaves in relation to the root system is prone to drought stress because the leaves lose water faster than the roots can supply. Newly transplanted plants and poorly established plants may be especially susceptible to drought stress because of a limited root system or a large mass of stem and leaves in comparison to roots.

## 37.3 PRACTICING LANDSCAPING UNDER LOW WATER AVAILABILITY SITUATIONS

To cope with stress faced by landscape plants under drought, a careful planning for an appropriate design and management strategy has a crucial role to play. The following approaches are followed to deal with the issues of water scarcity for landscaping:

1. Use of appropriate design
2. Selection of suitable plant species
3. Use of efficient irrigation systems
4. Use of alternative water resources
5. Use of appropriate management strategies of establishment and subsequent maintenance

### 37.3.1 APPROPRIATE PLANNING AND DESIGN

Landscape designers should look for ways to minimize the requirement of irrigation water and to maximize the use of natural precipitation [32]. In an attempt to reduce excessive water use, xeriscape is the most exciting concept to hit the landscape industry in decades. Xeriscape basically comes from a combination of two words: “xeri,” which is derived from the Greek word “xeros” meaning dry; and “scape,” meaning view or scene; together they mean “a dry scene.” The term xeriscape was coined in Denver, Colorado, in 1978 [33]. Xeriscape is an idea of quality landscaping that conserves water and protects the environment. Traditional landscapes usually incorporate one or two principles of water conservation, but they do not utilize the entire xeriscape concept to reduce landscape water use effectively [34].

Xeriscape practices have long been advocated by landscape architects, landscape designers, and horticulturists with little adoption [35], while many people still confuse xeriscaping with “zero-scaping.” Though both of these landscapes use less water than the traditional ones, these are totally different in appearance and appeal. Zero-scaping is the landscape that consists mostly of concrete, stones, or gravel, with perhaps a cactus or dull plants, while xeriscaping can look quite lush, cool, colorful, and full of beautiful plants like any other landscape but maintained with water-efficient practices. For a successful sustainable landscape, it takes an adjustment in the expectations of how a landscape should look like. What is gained is a more natural landscape that uses less labor and chemicals. It requires a much more careful planning than traditional landscapes, but establishing plant communities creates a landscape that becomes easier to maintain as it matures, and is self-perpetuating through proper plant selection.

#### 37.3.1.1 Orientation and Site Screening

Site orientation for north, south, east, and west is essential to mark for areas of sun and shade, which will help to establish zones of differing water needs. The wind current and strength in this kind of landscape is also an essential consideration, because high wind velocity raises the rate of evapotranspiration, resulting in an increase in the water requirements of plants, while plants or other elements such as walls or fences can be used as a windscreen.

#### 37.3.1.2 Contour Management

For utilizing any “limiting features,” such as existing trees, fences, walkways, or structures, it is necessary to study the natural contours and drainage patterns of the land. These contours can be easily developed into terraces, which add visual interest and help to reduce soil loss and erosion due to water runoff. Terraces can be as little as 3 in. and still offer visual appeal; terraces over 12 in. will require considerable support, such as rock walls or timbers reinforced with steel stakes [32]. The contours could be managed effectively by transforming the shape of the slope, keeping in view that a steep slope is ineffective to reduce water runoff while a gentle slope is desirable to hold water.

#### 37.3.1.3 Water Harvesting

Water harvesting is relatively a simple approach that works best for significant plant growth [36]. Rainwater is superior to other sources because it is free of salts and other minerals that may harm plant growth. As rainwater percolates into the soil, it forces excessive salts to leach down, away from root zones, allowing roots to grow better and making plants withstand the drought situation [37]. A good design at the household level to harvest rainwater usually consists of a catchment area, means of distribution, which operates by gravity, and a landscape-holding area or water storage area (Figure 37.1). The catchment areas should have a smooth hard surface, such as a rooftop or driveway. The distribution system connects the catchment area to the landscape-holding or water storage areas. Examples include gutters, channels, ponds, and swales. The landscape-holding areas store water in the soil for direct use by the plants that can be a tree base or plant island.



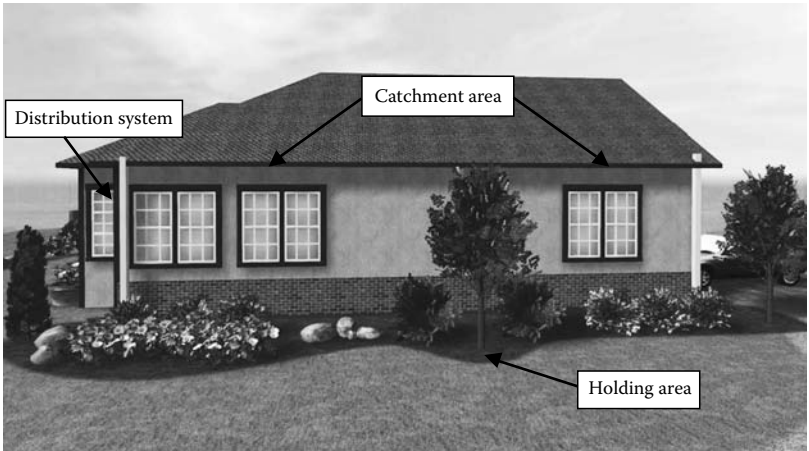


FIGURE 37.1 A simple system of rain water harvesting.

37.3.1.4 Area Allocation for Landscape Features and Utility Lines Demarcation

For small areas at home or large public parks and playgrounds, the planned use of each space within the area should be considered. Areas for seating, walkways, visual barriers, dinning, or play may also be defined and incorporated into the plan if required. It is necessary to plot all utility lines on the map in any case; this will help in plant division and allocation in the area.

37.3.1.5 Grouping the Plants according to Their Water Needs

It is necessary to dole out plant groups in design with similar watering needs for most efficient water use, because plants on the same irrigation set will not be under- or overwatered at the expense of other plants [38]. The area can be divided into high, moderate, and low water requirement zones [39,40] (Figure 37.2). Plants with shallow roots require less but frequent watering compared to deep-rooted plants, whereas plants with deep roots require more amount of water but may be with more intervals, in which case light irrigation will not penetrate deep down to the root zone, rather water will evaporate from the soil surface [41]. Plants should also be prioritized according to their

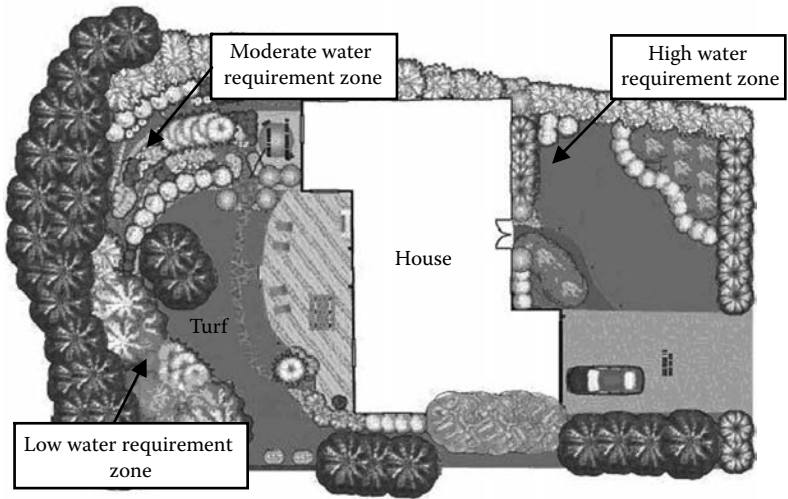


FIGURE 37.2 Area divided into high, moderate, and low water requirement zones.

susceptibility to water stress. High on the watering list should be plants that are valuable in terms of replacement cost and prominence in the landscape.

#### 37.3.1.6 Reduced Turf Area

One of the most controversial and misunderstood of the xeriscape principles is the concept of appropriate turf [42]. The size of turf areas should be reduced as much as possible in design, while retaining some turf for open space, functionality, and visual appeal. Lawn areas can be replaced with less-water-demanding landscapes, such as native gardens and shrubs. Areas to be left as turf should be designed to be easily mowed. Curved swaths are usually better than straight runs with sharp turns. Narrow swaths should be avoided because it can be difficult to water with conventional sprinklers [32].

#### 37.3.1.7 Expanded Planting Beds

It is better to increase the size of planting beds and develop planting islands in the grass area. Trees and shrubs can be planted in spacious, sweeping beds, rather than individually. Existing trees and shrubs can also be linked together as planting islands by adding an additional tree or two and replacing the lawn area between them with mulch or ground covers. In sunnier spots, “mulch islands” can be established, using ornamental grasses, showy perennials, and hardy native plants. Eventually, over a period of time, these individual “islands” can become the dominant landscape feature, with lawn areas that will serve as easily managed green lakes and open spaces among a more natural, graceful, and beautiful setting [43].

### 37.3.2 USE OF APPROPRIATE PLANT SPECIES

Appropriate plant selection is another important attribute to be considered for such landscapes. Plants which require less watering, pruning, spraying, fertilizer application and are resistant to pests and diseases should be selected for water-deficit conditions. It is necessary to select the right plant for the right site according to its light, soil, water, and humidity requirements and hardiness. Information of mature size, growth patterns, growth rate, and life span before planting will also help in long-term sustainability. It is better to select plants for their ultimate size. This will reduce pruning maintenance. For hot dry areas with south and west exposure, it is better to use plants that need only a minimum of water. Along north- and east-facing slopes and walls, plants that like more moisture will do good. Trees around the landscape help to reduce evaporation by blocking the wind and shading the soil. For best results, select plants that are native to the region and are drought resistant [32] especially in low-maintenance areas, because local native plants are typically hardier and more pest resistant, requiring less maintenance, and fertilizer and pesticide use [44], and are well adapted to local ecology.

Plants' ability to resist/tolerate drought under a given set of environmental conditions varies from species to species [45]. Plants may do this through drought avoidance, physiological adaptations that afford tolerance, or through efficiency mechanisms. However, some species have inherent anatomical or physiological characteristics to withstand drought because they have evolved in arid areas, regions with frequent drought, or with soils of low water-holding capacity. Different plants possess different characteristics to cope with drought conditions and can be categorized according to these characteristics. Consequently, the biological basis for drought tolerance is still largely unknown and few drought-tolerance determinants have been identified [12,46–48].

In general, drought-resistant plants are evergreen plants and have certain leaf adaptations to sustain growth under stress conditions. They include plant species having leaves that are long and narrow (e.g., *Helictotrichon sempervirens* and *Phormiums*), and small leaves (*Rosemary* and *Thymus*) [49] that shed heat, reduce the amount of surface area exposed to the atmosphere, and have few pores to lose water. Plants with thick or fleshy leaves store moisture for dry spells; *Sedum spectabile* (ice plant) is an example. Glossy (in *Salvia officinalis*) and silver-gray leaves (in *Lavender*) also

signify drought resistance. Fuzzy leaves of *Verbascum bombyciferum* are another means of reducing evaporation, in which case leaves shade themselves with their own hair and reduce air movement at the leaf surface. While many drought-tolerant species (*Pittosporum*, *Acacia*, and *Eucalyptus*) have developed exceptionally shiny thick cuticles that reduce the amount of water lost by evaporation from the leaf surface, particularly in windy conditions, some species have evolved large surface root systems to quickly absorb rainfall. Other species may grow deep root systems to tap deep water tables. Some plants avoid drought by dropping their leaves during droughts or hot summer and quickly regrowing new leaves when environmental conditions improve during winter water cycle (e.g., California paeonie) [31]. Some native annuals in different regions with short lifecycles disperse seeds and die, thus completing their lifecycle before drought sets in (e.g., California poppy and Lupine). Plants with immense water storage capacity, e.g., California fan palm, *Cycas revoluta*, Iris, Amaryllis, African lily, Callas, and Portulaca, are also tolerant to dry and hot conditions [50].

### 37.3.2.1 List of Drought-Tolerant Plants

As mentioned before, ornamental plant species that can be used in landscape vary greatly in tolerance and resistance to drought. The selection of suitable plant species for a given landscape scenario will depend on the “on-site” land, water, and environmental characteristics as well as on the objectives of landscape. Long lists of drought-tolerant plants are available in literature [51–53]. However, it is important to draw a sublist out of the literature of species suitable to different water regimes. The following list gives a spectrum of different types of landscape drought-tolerant plant species. Plants listed below (Table 37.1) by common and scientific names (alphabetized by common name) are grouped as vines, perennials, groundcovers, shrubs, trees, and annuals, and are further divided into categories such as S = slightly tolerant, M = moderately tolerant, and H = highly tolerant [54].

### 37.3.2.2 Drought-Tolerant Turfgrasses

For using turf in the landscape, more drought-tolerant species should be considered. Warm season grasses can be used where possible. Cool season grasses typically use 30% more water than warm season grasses. Grasses with excellent drought tolerance include Bahiagrass, Bermudagrass, and Zoysiagrass. Centipedegrass also possesses a good drought tolerance, while St. Augustine grass has a fair rating [55].

### 37.3.3 USE OF EFFICIENT IRRIGATION SYSTEM

Water conservation is the main goal of any water-efficient landscape, so overwatering must be discouraged. The application of water in xeriscaping does not necessarily mean using less water, but using available water more efficiently. For this purpose, landscape can be divided mainly into ornamentals and turf areas according to water requirements. Several irrigation systems have also been developed to save excessive use of water in landscape, including bubbler and drip/trickle irrigation systems for trees, shrubs, and ground cover, whereas the sprinkler system is quite useful for grasses. Drip irrigation has evolved considerably over the past 15 years or so, when perhaps only a professional could be trusted to install an appropriate and efficient system. Today, numerous manufacturers produce complete do-it-yourself kits, provide helpful telephone assistance, and, in some cases, even take plan of the garden and custom design a complete system. Moreover, while they lack many of the features of a true drip system, there are inexpensive soaker hoses (sometimes called membrane soaker hoses, since the whole hose surface is porous), which can be easily put in place or moved around the garden as needed. Some systems, particularly those with self-cleaning emitters, flow regulators, and fertilizer injectors, are obviously more expensive than a sprinkler. However, the environmental benefits and long-term cost savings can be considerable [56].

Both soaker hoses and drip irrigation systems offer the easiest and most efficient watering for landscapes, with flow rates that can be readily controlled to meet specific plant needs, while normally providing optimum irrigation using 75% less water, conserving water and saving on water bills, as well as conserving the energy required for treating and pumping the water to landscapes [56]. Furthermore,

**TABLE 37.1**  
**Drought-Tolerant Ornamental Plants**

| Common Name                           | Botanical Name                  | Tolerance |
|---------------------------------------|---------------------------------|-----------|
| <i>Drought-tolerant vines</i>         |                                 |           |
| Almanda                               | <i>Allamanda cathartica</i>     | M         |
| Anemone clematis                      | <i>Clematis montana</i>         | M         |
| Antigonum                             | <i>Antigonum leptopus</i>       | H         |
| Asparagus                             | <i>Asparagus falcatus</i>       | H         |
| Bougainvillea                         | <i>Bougainvillea</i> spp.       | H         |
| Common trumpet creeper                | <i>Campis radicans</i>          | M         |
| Creeping fig                          | <i>Ficus pumila</i>             | M         |
| Creeping juniper                      | <i>Juniperus horizontalis</i>   | M         |
| Cup of gold vine                      | <i>Solandra maxima</i>          | H         |
| Honeysuckle                           | <i>Lonicera sempervirens</i>    | H         |
| Morning glory                         | <i>Ipomoea</i> spp.             | M         |
| Rangoon creeper                       | <i>Quisqualis indica</i>        | M         |
| Senecio                               | <i>Senecio confusus</i>         | M         |
| Tecoma                                | <i>Tecoma grandiflora</i>       | H         |
| Wisteria                              | <i>Wisteria</i> spp.            | M         |
| <i>Drought-tolerant perennials</i>    |                                 |           |
| Blanket flower                        | <i>Gaillardia grandiflora</i>   | H         |
| Cactus                                | Various plant groups            | H         |
| Daylily                               | <i>Hemerocallis hybrids</i>     | S         |
| Lavender                              | <i>Lavendula angustifolia</i>   | M         |
| Sea lavender                          | <i>Limonium perezii</i>         | M         |
| Spurge                                | <i>Euphorbia</i> spp.           | H         |
| Stonecrop                             | <i>Sedum</i> spp.               | H         |
| Tickseed                              | <i>Coreopsis</i> spp.           | H         |
| Vinca                                 | <i>Vinca rosea</i>              | H         |
| <i>Drought-tolerant ground covers</i> |                                 |           |
| English lavender                      | <i>Lavandula angustifolia</i>   | M         |
| Ivy geranium                          | <i>Pelargonium peltatum</i>     | M         |
| Purple heart                          | <i>Setcreasea pallida</i>       | H         |
| Ramanas rose                          | <i>Rosa rugosa</i>              | H         |
| Rosemary                              | <i>Rosemarinus officinalis</i>  | M         |
| Russelia                              | <i>Russelia juncea</i>          | H         |
| Trailing African daisy                | <i>Osteospermum fruticosum</i>  | H         |
| Trailing ice plant                    | <i>Lampranthus spectabilis</i>  | H         |
| Wandering jew                         | <i>Tradescantia fluminensis</i> | H         |
| <i>Drought-tolerant shrubs</i>        |                                 |           |
| Alpinia                               | <i>Alpinia caerulea</i>         | H         |
| Butterfly bush                        | <i>Buddleia davidii</i>         | H         |
| California lilac                      | <i>Ceanothus concha</i>         | H         |
| Common lilac                          | <i>Syringa vulgaris</i>         | H         |
| Crape myrtle                          | <i>Lagerstroemia indica</i>     | H         |
| Creeping coprosma                     | <i>Coprosma kirkii</i>          | H         |
| Desert rose                           | <i>Adenium obesum</i>           | H         |
| Heavenly bamboo                       | <i>Nandina domestica</i>        | H         |

(continued)

**TABLE 37.1 (continued)**  
**Drought-Tolerant Ornamental Plants**

| Common Name                   | Botanical Name                      | Tolerance |
|-------------------------------|-------------------------------------|-----------|
| Henna                         | <i>Lawsonia inermis</i>             | H         |
| Hopseed bush                  | <i>Dodonaea viscosa</i>             | H         |
| Indian hawthorne              | <i>Raphiolepis indica</i>           | M         |
| Jatropha                      | <i>Jatropha curcas</i>              | H         |
| Lantana                       | <i>Lantana camara</i>               | M         |
| Natal plum                    | <i>Carissa grandiflora</i>          | M         |
| Oleander                      | <i>Nerium oleander</i>              | H         |
| Prostrate acacia              | <i>Acacia redolens</i>              | H         |
| Rockrose                      | <i>Cistus hybrids</i>               | H         |
| Salt bush                     | <i>Atriplex halimus</i>             | H         |
| Senecio                       | <i>Senecio greyii</i>               | H         |
| Spiraea                       | <i>Spiraea</i> spp.                 | M         |
| Toyon                         | <i>Heteromeles arbutifolia</i>      | H         |
| Wormwood                      | <i>Artemisia absinthium</i>         | H         |
| Yellow oleander               | <i>Thevetia nerifolia</i>           | H         |
| Yellow trumpet bush           | <i>Tecoma stans</i>                 | H         |
| Yucca                         | <i>Yucca</i> spp.                   | H         |
| <i>Drought-tolerant trees</i> |                                     |           |
| Acacia                        | <i>Acacia</i> spp.                  | H         |
| Aleppo pine                   | <i>Pinus halepensis</i>             | H         |
| Australian willow             | <i>Geijera parviflora</i>           | M         |
| Bottle brush                  | <i>Callistemon</i> spp.             | H         |
| Canary island pine            | <i>Pinus canariensis</i>            | H         |
| Catalina ironwood             | <i>Lyonathammus floribundus</i>     | M         |
| Common fig                    | <i>Ficus carica</i>                 | H         |
| Eucalyptus                    | <i>Eucalyptus</i> spp.              | H         |
| Ficus                         | <i>Ficus nitida</i>                 | M         |
| Floss silk tree               | <i>Chorisia speciosa</i>            | H         |
| Golden shower tree            | <i>Cassia fistula</i>               | H         |
| Ironwood                      | <i>Casuarina equisetifolia</i>      | H         |
| Jacaranda                     | <i>Jacaranda mimosifolia</i>        | M         |
| Jerusalem thorn               | <i>Parkinsonia aculeata</i>         | H         |
| Jujube                        | <i>Ziziphus jujuba</i>              | H         |
| Kanakchampa/karnikara         | <i>Pterospermum acerifolium</i>     | H         |
| Lemon bottle brush            | <i>Callistemon citrinus</i>         | H         |
| Lemon gum                     | <i>Eucalyptus citriodora</i>        | H         |
| Life tree/jiaputra            | <i>Putranjiva roxburghii</i>        | H         |
| Mexican fan palm              | <i>Washingtonia robusta</i>         | H         |
| Pink melaleuca                | <i>Melaleuca nesophila</i>          | M         |
| Pongamia                      | <i>Pongamia glabra</i>              | H         |
| Poplar                        | <i>Populus alba</i>                 | H         |
| Prosopis                      | <i>Prosopis</i> spp.                | H         |
| Salt cedar                    | <i>Tamarix aphylla</i>              | H         |
| Siris tree                    | <i>Albizia lebbek</i>               | H         |
| Tooth brush tree              | <i>Salvadora persica</i>            | H         |
| Waras                         | <i>Heterophragma quadriloculare</i> | H         |
| White willow                  | <i>Salix alba</i>                   | M         |

**TABLE 37.1 (continued)**  
**Drought-Tolerant Ornamental Plants**

| Common Name                           | Botanical Name                | Tolerance |
|---------------------------------------|-------------------------------|-----------|
| <i>Drought-tolerant annual plants</i> |                               |           |
| Candytuft                             | <i>Iberis sempervirens</i>    | M         |
| Calendula                             | <i>Calendula officinalis</i>  | M         |
| Celosia                               | <i>Celosia</i> spp.           | H         |
| Centaurea                             | <i>Centaurea montana</i>      | H         |
| Cosmos                                | <i>Cosmos bipinnatus</i>      | H         |
| Dusty miller                          | <i>Senecio cineraria</i>      | H         |
| Gazania                               | <i>Gazania maritima</i>       | M         |
| Gazania                               | <i>Gazania rigens</i>         | H         |
| Globe amaranth                        | <i>Gomphrena globosa</i>      | H         |
| Hollyhock                             | <i>Althaea rosea</i>          | H         |
| Lily of the Nile                      | <i>Agapanthus africanus</i>   | M         |
| Marigold                              | <i>Tagetes</i> spp.           | M         |
| Mexican evening primrose              | <i>Oenothera berlandieri</i>  | M         |
| Morning glory                         | <i>Ipomoea purpurea</i>       | M         |
| Moss rose, Rock rose                  | <i>Portulaca grandiflora</i>  | H         |
| Nasturtium                            | <i>Tropaeolum majus</i>       | M         |
| Pinks                                 | <i>Dianthus barbatus</i>      | M         |
| Sage                                  | <i>Salvia nemerosa</i>        | M         |
| Statice                               | <i>Statice limonium</i>       | H         |
| Straw flower                          | <i>Helichrysum bracteatum</i> | H         |
| Verbena                               | <i>Verbena hybrida</i>        | H         |
| Zinnia                                | <i>Zinnia elegance</i>        | M         |

because drip systems can be buried underground, the water goes only to the plant’s roots directly and more precisely, which reduces moisture loss from evaporation from soil, reduces weed growth, and prevents many of the diseases caused by over- or underwatering. They also deliver the water at a slow rate, which encourages root absorption and reduces pooling and minimizes runoff.

Like ornamental plants, turf irrigation also requires a well-designed irrigation system where the sprinkler is very much in use at various places including home lawns, golf courses, parks, etc. Technologies such as soil moisture sensor and irrigation controllers have been shown to reduce irrigation by 70%–90% over a range of irrigation schedules and controller brands [57].

### 37.3.4 USE OF ALTERNATIVE WATER RESOURCE FOR LANDSCAPING

In many situations of freshwater scarcity, alternative resources of irrigation are available. These resources primarily include saline water and treated/untreated water recovered from industry, household, or sewerage system.

#### 37.3.4.1 Use of Saline Water

Saline water generally contains an excessive concentration of total soluble salts having high amounts of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_3^{2-}$ . These waters have two types of effects on plants. First, due to the high concentration of salts, the osmotic potential (and total potential) of water is decreased to reduce the gradient between outside and inside the roots, thus hindering the water uptake by plants. This phenomenon is similar to drought effects and is sometimes termed as “physiological drought” of plants. Second, there are “specific ion effects” due to a high uptake of ions like  $\text{Na}^+$  and  $\text{Cl}^-$  and sometimes boron. These salts cause chlorosis and necrosis of plant leaves [58–62].

**TABLE 37.2**  
**Classification of Irrigation Water for Potential Salinity/Sodicity Hazards**

| Classification | EC (dS/m) | Sodium Adsorption Ratio (SAR) | Residual Sodium Carbonate (RSC) |
|----------------|-----------|-------------------------------|---------------------------------|
| Useable        | <1.5      | <15                           | <2.5                            |
| Marginal       | 1.5–3.0   | 15–18                         | 2.5–5                           |
| Hazardous      | >3.0      | >18                           | >5                              |

Source: Qureshi, R.H. and Barret-Lennard, E.G., *Saline Agriculture for Irrigated Land in Pakistan: A Hand Book*, Australian Centre for International Agriculture Research, Canberra, Australia. Better Printing, Queanbeyan, Australia, p. 141.

Note:  $RSC = [HCO_3^- + CO_3^{2-}] - [Ca^{2+} + Mg^{2+}]$ , where concentrations are in milliequivalents per liter.

Saline waters having excessive amounts of  $Na^+$ ,  $HCO_3^-$ , and  $CO_3^{2-}$  have additional effects by altering the soil structure, which breaks the granular structure and the soil becomes dense, causing problems of root penetration and aeration [63,64].

The water is generally categorized into various classes on the basis of potential soil hazards as under (Table 37.2).

Plant and soil scientists have classified plants with respect to tolerance not only to salinity but also to specific ions. Mass and Hoffman [66] classified plants on the basis of threshold salinity, at which the yield starts declining, and a 50% reduction in yield is observed compared with the yield under nonsaline conditions. The list of relatively salt-tolerant ornamental plants, showing tolerance against substrate salinity (H = highly tolerant, M = moderately tolerant, S = slightly tolerant), is given in Table 37.3 [67–69].

37.3.4.1.1 *Salt-Tolerant Grasses*

Zoysia, Common Bermuda, Seashore Paspalum, and Saltgrass (*Distichlis spicata*) are warm season turfgrass species. These grasses can be grown successfully in certain low-maintenance turfgrass areas, under highly saline conditions and limited available water sources [70–79].

The author has used highly saline/sodic water having EC = 4.3 dS/m and SAR = 22.9 for several years to maintain a healthy lawn and plants like *Alternanthera dentata* (ground cover), *Phoenix roebelenii* (palm), *Ficus benjamina* (Plate 37.2), *Ficus natalensis*, Hibiscus, *Dracaena* (tree/shrub), etc.

37.3.4.1.2 *Methods of Using Saline Water for Irrigation*

The harmful effects of using poor quality water for landscape plants can be mitigated in a number of ways, as is done for agronomic crops:

1. Dilution

Saline water may be mixed with freshwater/sewage water, etc., in various ratios to bring it to within safe limits with respect to its effect on soil and plants.

2. Alternative use with freshwater

Depending upon the availability of freshwater, saline water can be used at various intervals for irrigation, e.g., after every one, two, or three irrigations with freshwater.

3. Use of amendments

The addition of amendments like gypsum ( $CaSO_4 \cdot 2H_2O$ ) and sulfur greatly mitigate the deleterious effect of  $Na^+$  on soil and also on plants. Calcium not only improves the soil structure, but also improves the cell membrane integrity and helps in the selective uptake of  $K^+$  against  $Na^+$ .

**TABLE 37.3**  
**Salt-Tolerant Ornamental Plants**

| Common Name                         | Botanical Name                       | Salt Tolerance |
|-------------------------------------|--------------------------------------|----------------|
| <i>Salt-tolerant shrubs</i>         |                                      |                |
| Azalea                              | <i>Rhododendron</i> spp.             | S              |
| Butcher's broom                     | <i>Ruscus aculeatus</i>              | H              |
| Century plant                       | <i>Agave americana</i>               | H              |
| China rose                          | <i>Hibiscus syriacus</i>             | H              |
| Firebush                            | <i>Hamelia patens</i>                | M              |
| Hydrangea                           | <i>Hydrangea</i> spp.                | M              |
| Indian hawthorne                    | <i>Raphiolepis indica</i>            | M              |
| Japanese euonymus                   | <i>Euonymus japonicus</i>            | M              |
| Japanese privet                     | <i>Ligustrum japonicum</i>           | M              |
| Oleander                            | <i>Nerium oleander</i>               | H              |
| Pittosporum                         | <i>Pittosporum tobira</i>            | H              |
| Rosemary                            | <i>Rosmarinus officinalis</i>        | H              |
| Saltbush                            | <i>Atriplex</i> spp.                 | H              |
| St. Johnswort                       | <i>Hypericum</i> spp.                | M              |
| Yucca                               | <i>Yucca</i> spp.                    | H              |
| <i>Salt-tolerant vines/creepers</i> |                                      |                |
| Bougainvillea                       | <i>Bougainvillea</i> spp.            | H              |
| Creeping fig                        | <i>Ficus pumila</i>                  | M              |
| English ivy                         | <i>Hedera helix</i>                  | M              |
| Honeysuckle                         | <i>Lonicera japonica</i>             | M              |
| Star jasmine                        | <i>Trachelospermum asiaticum</i>     | M              |
| <i>Salt-tolerant ground covers</i>  |                                      |                |
| Agapanthus                          | <i>Agapanthus africanus</i>          | M              |
| Blanket flower                      | <i>Gaillardia pulchella</i>          | H              |
| Cast iron plant                     | <i>Aspidistra elatior</i>            | M              |
| Creeping juniper                    | <i>Juniperus horizontalis</i>        | M              |
| Daylily                             | <i>Hemerocallis species</i>          | M              |
| Golden stonecrop                    | <i>Sedum acre</i>                    | H              |
| Mondograss                          | <i>Ophiopogon japonicus</i>          | H              |
| Vinca                               | <i>Vinca minor</i>                   | M              |
| Prickly pear cactus                 | <i>Opuntia compressa</i>             | H              |
| Purple heart                        | <i>Setcreasea pallida</i>            | M              |
| Winter creeper                      | <i>Euonymus fortunei</i>             | H              |
| <i>Salt-tolerant annuals</i>        |                                      |                |
| Coleus                              | <i>Solenostemon hybrids</i>          | S              |
| Ice plant                           | <i>Mesembryanthemum crystallinum</i> | H              |
| Marigold                            | <i>Tagetes erecta</i>                | S              |
| Moss rose                           | <i>Portulaca grandiflora</i>         | H              |
| <i>Salt-tolerant trees</i>          |                                      |                |
| American holly                      | <i>Ilex opaca</i>                    | M              |
| Arizona cypress                     | <i>Cupressus arizonica</i>           | M              |
| Black wattle                        | <i>Acacia meamsii</i>                | H              |
| Crape myrtle                        | <i>Lagerstroemia hybrids</i>         | M              |

(continued)



**TABLE 37.3 (continued)**  
**Salt-Tolerant Ornamental Plants**

| Common Name         | Botanical Name               | Salt Tolerance |
|---------------------|------------------------------|----------------|
| Eucalyptus          | <i>Eucalyptus citriodora</i> | H              |
| Golden wattle       | <i>Acacia longifolia</i>     | H              |
| Japanese black pine | <i>Pinus thunbergii</i>      | H              |
| Live oak            | <i>Quercus virginiana</i>    | H              |
| Lombardy poplar     | <i>Populus nigra</i>         | H              |
| Magnolia            | <i>Magnolia grandiflora</i>  | H              |
| Pongamia            | <i>Pongamia glabra</i>       | H              |
| Tamarix             | <i>Tamarix ramosissima</i>   | M              |
| Weeping willow      | <i>Salix alba</i>            | H              |



**PLATE 37.2** Ornamental plants grown with highly saline/sodic water.

4. Use of trickle/bubble methods of irrigation
- Irrigations using trickle/bubble systems minimize the accumulation of salts near the plant roots by pushing them to the water front away from the point of water addition. The use of saline water by sprinkle irrigation should be avoided as direct spraying of saline water on leaves may cause salt injury, leading to an unpleasant look of the ornamental plants.

**37.3.4.2 Use of Reclaimed Water**

Reclaimed water is the non-potable wastewater effluent that has been previously treated [80] and contains higher levels of nutrients than potable water. The use of reclaimed water to irrigate landscapes has increased in recent years. One of the most important benefits of using reclaimed water for irrigation in landscapes is that it spares potable water for other human needs [81]. Reclaimed water can be used for irrigating residential landscapes, golf courses, playgrounds, public school yards, and parks after treatment, while, due to high-level disinfection, it is not considered a threat to public health [82,83]. Special management practices may be required to deal with the salts present in reclaimed water, depending on the quantity and kind of salts and the salt sensitivity of the plants to be irrigated. Nutrients supplied by reclaimed water also minimize the need for additional fertilizers. Nutrient supply through reclaimed water to golf courses has been recommended by several turfgrass experts [84–87].

## 37.4 APPROPRIATE STRATEGIES OF LANDSCAPE ESTABLISHMENT AND SUBSEQUENT MAINTENANCE

### 37.4.1 MANAGEMENT DURING ESTABLISHMENT OF LANDSCAPES

#### 37.4.1.1 Soil Improvement

Before the start of execution of a landscape plan, it is required to manage the soil. The ideal soil for a water-conserving landscape does two things simultaneously, i.e., it drains quickly but holds sufficient water at the same time. This can be achieved by increasing the amount of organic matter in the soil, which will also keep it well aerated. Compost is the ideal organic additive, unless the design contains many succulents and cacti. Clean and sterilized leaf compost, farm yard manure, or peat moss can ideally be used to increase organic matter and fertility in the soil.

#### 37.4.1.2 Mass Plantings

Similar to planting islands for trees, it is best to mass plants together, rather than spreading them across a broad area. Massed plantings have a stronger visual impact than a row of annuals dotted in front of a shrubbery. Moreover, by grouping plants together according to similar water needs, they can be cared for much more easily, and can more readily care for themselves. A thick, established group of plants will keep out weeds and will shade the soil around their root zones, thereby conserving precious moisture and reducing drastic changes in soil temperature [43].

#### 37.4.1.3 Irrigation System

##### 37.4.1.3.1 *Ornamentals*

Ornamental plants should again be divided into different zones according to their water needs. Trees may occupy low-water-use zones, whereas shrubs, perennials, and other planting beds usually occupy low- to medium-water-use zones, partly depending on the season and growth rate. Watering by hand in these areas is a tricky and time-consuming business, requiring more water, while applying water unevenly or dampening foliage at midday or in the evening can often lead to fungal diseases and other problems. These plantings are best served with a drip irrigation system, or soaker hoses, which are less expensive than the drip irrigation system [56].

Most large landscape plants require 1 in. of water per week during the growing season. This is equivalent to approximately 750 gal of water per 1000 ft<sup>2</sup> beneath the crown. For new transplants, trees with damaged roots, or plants growing in sandy soil, water should be provided at least twice a week. Water should be concentrated on the root ball of new plantings. On established plantings in clay or loam soils, the recommended quantity of water should be supplied at least once a week.

##### 37.4.1.3.2 *Turfs*

All turfgrasses need water to sustain good quality (dense, uniform green looks), whether it comes from rainfall or supplemental irrigation. Newly installed turfgrass requires light irrigation two to three times a day for the first 7–10 days, once a day for the following 7 days, followed by every other day for another 7 days. After 30 days, when the turf establishes, irrigation should occur based on the plant response to the environmental demand. Turfgrasses generally require 1 in. of water per week during the growing season for optimum growth [88]. Thus, the irrigation frequency and amount will be defined by the environmental demand (i.e., evapotranspiration, soil water-holding capacity, and plant root zone depth). On sandy soils, some grasses may need to be irrigated at least 2 days a week to ensure acceptable quality [89], while, in hot summer, they may require even more water. More frequent and shallow watering can cause thatch and shallow drought-sensitive roots. An established turf needs irrigation only after about 30% of the lawn

starts to wilt. Signs of wilting include footprints that remain in the grass long after being made, a bluish-gray appearance of the lawn, and a large proportion of leaf blades that are folded in half lengthwise. Generally,  $\frac{1}{2}$ – $\frac{3}{4}$  in. of irrigation is recommended when 30%–50% of turfgrass shows signs of wilt during the day.

Normally, lawns require about 1 in. of water, although no more than once a week. To measure the amount of water being applied through a sprinkler, a flat pan can be placed under the sprinkler until 1 in. of water has accumulated. It is to be ensured that the soil is moistened to a depth of 4–6 in. by pushing a screwdriver into the ground as an indicator. The hose should be turned off if water starts to spill onto paved areas, and watering should be resumed after waiting for 30 min [90].

#### 37.4.1.3.3 *Irrigation Frequency*

Newly installed plants require frequent irrigations to become established. Recently planted shrubs and trees can survive on two or three weekly applications. Recently planted trees, up to a 3 in. trunk diameter, can survive on 2–3 gal of irrigation per inch of trunk diameter applied to the root ball two to three times weekly. However, they grow best with more frequent irrigation until fully established. Establishment takes 3–4 months per inch of trunk diameter, whereas shrubs planted from 3 gal containers can be established by applying 1 gal of water every other day. This regime provides for good root growth and some top growth during establishment [90].

#### 37.4.1.4 **Mulching**

Mulching is covering the soil surface around plants with materials such as leaves, coarse compost, pine needles, wood chips, shredded bark, bark nuggets, or gravel. Mulches can prevent up to 73% evaporation loss, reduce weed growth, prevent soil erosion, even out variations in the soil temperature over the day and night, and improve the soil structure. Wood chips from tree-pruning operations are particularly effective and inexpensive mulches.

Mulching with organic materials is one of the easiest and conducive methods for mulching, which slowly incorporates and adds organic matter to the soil for conserving soil moisture, promoting root development, and providing long-term soil improvement. These kind of mulches need frequent applications, i.e., topdressing, from time to time. There should be no areas of bare soil. To be effective, mulches should be applied to a depth of 2–4 in. around landscape plants except over shallow-rooted plants like azaleas. This depth should not be exceeded around trees, as this could be detrimental. Preferably, mulches should be applied to the “dripline” of the plant whenever possible. However, a narrow mulch ring around plants is better than none. Mulches should not be applied against the stem and root collar of plantings. Keeping the weeds from growing up through the mulch may require some attention, for which thickening the layer of the mulch will help [91].

After applying the mulch, especially when using wood chips or materials that appear dry, it is advisable to water both mulch and plants thoroughly at first. Dry mulch might otherwise keep moisture from percolating into the soil. Woody mulches are best used around permanent plantings like trees and shrubs, while finer-textured mulches, such as untreated grass clippings, compost, shredded leaves, and leaf mold, are preferable for tender plantings, such as annual and perennial flowering plants [43].

### 37.4.2 **MAINTENANCE AND MANAGEMENT OF LANDSCAPES AFTER ESTABLISHMENT**

Low maintenance is one of the benefits of xeriscape, but a properly designed landscape still requires consistent maintenance, although most of the maintenance can be avoided by thorough planning and design, appropriate installation techniques, and timely observations of the condition of grasses, plants and their beds, and irrigation system components.

#### 37.4.2.1 **Irrigation**

Irrigation schedules developed for turfgrass can over-irrigate ornamental plantings. The most efficient way to irrigate trees and shrubs is using micro-irrigation. Ornamental plants require more

water to go deep in the root area of plants as compared to grass, and it can be with more intervals. Similarly, among ornamentals, plants with a shallow root system require less but frequent water as compared to deep-rooted plants. Moreover, in the case of newly planted trees and shrubs, water should be applied to the root ball and perhaps the soil just beyond the root ball. Trees and shrubs can establish fine without broad, landscape-wide (i.e., sprinkler) irrigation, e.g., live oak and magnolia tree root systems extend to about 14–20 ft in diameter 1 year after planting in a non-compacted soil without interference from curbs, sidewalks, and other soil obstructions. The irrigation system should also be examined periodically, and leakage should be repaired promptly.

#### *37.4.2.1.1 Irrigation Priorities*

Newly installed plants, and highly visible and intensively managed areas should be the first priority. Further, drought-sensitive and wilting plants should have the high-priority consistency among all. Turf should have lower priority. Although turf may be drought sensitive, it is cheaper to replace turf than to replace trees and shrubs. Drought-tolerant plants, such as many established trees and shrubs and turfgrass, may not need irrigation in the beginning stages of drought but need water when plants start showing stress signs. Infrequent rains during a drought often help to provide enough water to keep these plants alive.

#### *37.4.2.1.2 Time of the Day*

Watering should be done only in the early morning, because less water is lost to evaporation and wind drift in the morning because of cooler temperatures and less wind. Watering during the day or in the evening should be avoided. Improper watering can lead to fungal diseases or scalded foliage.

#### *37.4.2.1.3 Irrigation Frequency*

Extended drought can cause a decline in even drought-tolerant established large trees. Once established, shrubs and trees do not require frequent irrigation, unless they show signs of stress, such as wilting leaves, change in leaf color, yellowing leaves, or dropping leaves. For established plants, enough irrigation needs to be applied to wet the soil deep rather than light amounts that wet only the surface. Deep watering provides water to a larger portion of the root system. Deep, infrequent irrigation could also improve drought resistance by promoting deeper, more extensive root systems in some cases. Soil depth aimed for watering should be 6–12 in. for turf and bedding plants, and 12 in. for perennials, shrubs, and trees. A depth of 12 in. is achieved with 1 in. of irrigation on sandy soils and may be shallower for clay soils. Many trees and shrubs can survive drought without irrigation, provided that they are well established and were irrigated prior to the drought [90].

### **37.4.2.2 Weed Control**

Keeping weeds under control, either by manual pulling or using some suitable weedicide, helps in saving water. Weeds can steal a fair amount of water from plants [92] and nutrients. One should avoid the use of herbicides where possible and perform weed control manually by hand.

### **37.4.2.3 Pesticide Application**

Unnecessary applications of pesticides that require watering should be avoided.

### **37.4.2.4 Fertilization**

Maintaining adequate soil fertility helps to prevent nutrient stress and minimize the effects of drought. During drought, plants should not be fertilized, because fertilization stimulates growth and can increase water needs. The use of phosphorus and potash along with nitrogen before the drought season can be helpful in tolerating the drought later on. However, the use of compost or farm yard manure is a better option for improving soil fertility as well as physical conditions of the soil. Organic and slow-release fertilizers are generally the best for the growth of woody plants. Agricultural-grade fertilizers should be avoided, which may have a salt effect that can intensify

drought stress and can severely injure plants if applied to drier soils. Best results can be achieved with fertilizer treatments based on soil analysis results. Primarily, fertilizers should be applied after drought has ended and soils are recharged by rainfall. Native plants may need little or no fertilizer application.

#### 37.4.2.5 Pruning

During drought, dead and dying limbs on landscape plants should be removed. These limbs may harbor insect borers or canker disease fungi that can contribute to further dieback and decline. If crowns are very dense, light thinning can help to reduce the demand for water and nutrients. Shrubs can be pruned to reduce their size or thinning of canopy can be done to reduce the leaf area. Pruning should be carefully done because significant pruning of live branches will add additional stress from defoliation and wounding. Weak plants can be removed in beds by thinning, which will reduce competition among plants.

#### 37.4.2.6 Use of Antitranspirants

Antitranspirants are those materials applied as a spray to the foliage that act as a barrier to water loss. These materials can produce a short-term benefit by reducing transpirational water loss. Antitranspirants may be useful on recent transplants or when trees cannot be routinely irrigated for brief periods in summer.

#### 37.4.2.7 Pest Management

Insect pests and disease organisms weaken trees by defoliation or by causing stem and root damage that impedes absorption and translocation of water and nutrients. Some insects act as vectors for diseases. Some fungi infect the roots. Drought-stressed plants are particularly prone to pest infestations. Pests should be managed using integrated pest management (IPM) principles, a technique requiring periodical inspection of plants for pests. When detected, pests should be maintained below levels that impact plant health, through cultural, biological, and/or chemical treatments.

#### 37.4.2.8 Lawn Mowing

Frequent mowing increases the lawn water demand because it promotes new leaf growth. Lawns should not be mowed to less than 2–3 cm. This aids in moisture retention. Deeper root systems are encouraged through less frequent but heavier watering. This will make plants and lawns more drought resistant.

The cutting height of turf should be raised during the drought period. Although taller grass uses slightly more water than shorter grass, a higher cutting height promotes deeper rooting and maintains the turf quality longer.

Mowing frequency may be adjusted so that no more than one-third of the turfgrass leaf blade is removed at any one time. Under drought conditions, growth will be reduced, so the frequency may be reduced [90].

Also, it is necessary to use a sharp blade when mowing. A sharp mower blade produces a cleaner cut that heals more quickly and results in less water loss than a cut made by a dull blade.

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# 38 Turfgrass Nutrient Management under Stresses: A Part of Integrated Stress Management

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## 38.1 INTRODUCTION

“Turfgrass nutrient management under stresses” is not a new concept for turfgrass science and management (Beard, 1973; Busey, 2003; McCarty and Tucker, 2005). For turf practitioners, it has been used since a fertilizer program was implemented; for turf scientists, it has been investigated since turf research started. However, there is a lack of literature reviews focusing on systematic approaches using nutrient management to reduce stresses, and this chapter aims at such an approach.

Non-food and non-fiber grasses, including ornamental grasses and turfgrasses, are important to human beings (Beard and Green, 1994). These grasses came from their origins by natural spreading or artificial collections and require relatively less nutrient input in comparison with other agricultural crops with focuses on yield. Turfgrass has been well accepted by human beings since the 1300s or an earlier time (Beard, 2002). Today, home lawns show the closest relationship between human life and grasses—hundred millions of home lawns surrounding their homes on this earth. Most of these managed turfgrass areas experience an unfavorable stress such as drought, low fertility, poor soil conditions, temperature extremes, salinity, shade, low soil pH, traffic compaction, weed, disease, insect and mite pests, and others at least once per growing season (Liu et al., 2008b). Some may have to overcome multiple stresses at a time and some may have to face permanent stresses year-round, particularly associated with lower mowing heights and intensive turf use. Intensive studies related to turfgrasses, particularly in the past 50 years, have focused on the mentioned stresses for enhanced turfgrass management, turfgrass improvement, integrated pest management (IPM), and environmental stewardship, with which findings have been applied and adapted in turfgrass management.

The major turfgrass species are in the grass family, which includes major agricultural crops such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench], barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and other forage crops. By comparison with other crops, turfgrasses require relatively less nutrients, but fertilizers are still needed (Turner and Hummel, 1992; Carrow et al., 2001). Overall turf use and quality can be enhanced by proper fertilizer application, systematic management programs, and improved turfgrasses to overcome stressful conditions. Reviews on turfgrass nutrient use, physiology, and stress resistance and management have been well documented in the last decades (Dernoeden, 2000; McCarty and Miller, 2002; Fry and Huang, 2004; McCarty, 2011).

## 38.2 TURF STRESSES

In order to discuss the topic thoroughly, these stresses are grouped in to four subtopics: biotic stresses, abiotic stresses, turf use and management stresses, and pollution stresses. Turfgrass nutrient enhancement under these stresses can minimize the severity and longevity of stresses and even can be the critical factor under certain conditions. Stresses to turfgrasses are generated from two major sources, natural stresses such as temperature extremes, poor soil conditions, and pests, and non-natural stresses such as traffic, cultivation, and mowing (Table 38.1). The natural stresses can be divided into biotic and abiotic stresses. Biotic stresses are caused by living organisms such as weeds, diseases, insects and mites, and other living pests. Abiotic stresses are unfavorable growth conditions such as heat, cold, drought, flooding, salinity, acidic soils, nutrient deficiency, nutrient imbalance and toxicity, and excessive organic matter content. Non-natural stresses are mainly related to turf use including lower (or higher) mowing heights, cultivation, traffic, over-use, and other stresses caused by human activities. It is challenging to enhance nutrient use under those stressful conditions, and general maintenance strategies can be complex as well, including multiple approaches. Since basic practices of mowing, fertilizing, and watering can have significant impacts to a given stress or multiple stresses at a time, the definition of stress to turfgrasses is a concept covering all unfavorable conditions that cause negative impacts to turfgrass growth, performance, and function.

**TABLE 38.1**  
**General Relationships between Nutrients and Stresses for Turfgrasses**

| Stresses                      | Relationship between Management Level and Stresses  | Nutrients and Elements That May Have More Significant Impacts and Influences for the Severity of Stresses Than Others | Updated Evidences for Genetic Improvements | References                                                                                                                                                                                                      |
|-------------------------------|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Biotic stresses</b>        |                                                     |                                                                                                                       |                                            |                                                                                                                                                                                                                 |
| Weeds                         | Lower maintenance turf suffers more                 | N, P, K, and Na                                                                                                       | Unknown                                    | Busey (2003), McCarty and Tucker (2005)                                                                                                                                                                         |
| Diseases                      | Turf mown lower suffers more                        | N, Mn, Al, Cu, Zn, and Si                                                                                             | Existed and promising                      | Hill et al. (1999) and Datnoff (2005)                                                                                                                                                                           |
| Insects and mites             | All types                                           | N, P, and Si                                                                                                          | Existed and promising                      | Reinert and Engelke (2001)                                                                                                                                                                                      |
| <b>Environmental stresses</b> |                                                     |                                                                                                                       |                                            |                                                                                                                                                                                                                 |
| Drought                       | Cool-season and lower mowing turfgrasses            | N, K, Si, Ca, Fe, and Na                                                                                              | Existed and promising                      | Huang (2001), Karcher et al. (2008), Richardson et al. (2008), Su et al. (2008), and Li et al. (2010)                                                                                                           |
| Waterlogging                  | All types of turf                                   | Unknown                                                                                                               | Existed                                    | Jiang and Wang (2006)                                                                                                                                                                                           |
| Salinity                      | Cool-season turfgrasses and warm-season turfgrasses | K, Ca, and N                                                                                                          | Existed and very promising                 | Marcum and Murdoch (1994), Marcum (2001), Qian et al. (2001), Lee et al. (2004a,b), Baldwin et al. (2006), Marcum and Pessarakli (2006), Qian et al. (2007), and Li et al. (2010)                               |
| Heat                          | Cool-season turfgrasses                             | N, K, Ca, Fe, and Si                                                                                                  | Existed and promising                      | Zhang and Ervin (2008), Brecht et al. (2009), Xu and Huang (2009), and Zhang et al. (2010)                                                                                                                      |
| Low temperatures              | Warm-season turfgrasses and cool-season turfgrasses | N, K, Ca, Na, and Si                                                                                                  | Existed and promising                      | Anderson and Taliaferro (2002), Ebdon et al. (2002), Anderson et al. (2002, 2003), Munshaw et al. (2004), Webster and Ebdon (2005), Patton and Reicher (2007), Rukavina et al. (2007), and Dionne et al. (2010) |
| Shade                         | Bermuda grasses and others                          | N and Fe                                                                                                              | Existed                                    | Baldwin et al. (2008) and Sarvis et al. (2009)                                                                                                                                                                  |
| Acidic soils                  | All turfgrass species                               | N, Ca, and Fe                                                                                                         | Existed                                    | Murray and Foy (1978), Liu et al. (1995, 1996, 1997), Foy and Murray (1998), Baldwin et al. (2005), Liu (2005), and Yan et al. (2009)                                                                           |
| Nutrient imbalances           | All types of turf                                   | All nutrients                                                                                                         | Unknown                                    | Carrow et al. (2001)                                                                                                                                                                                            |

(continued)

**TABLE 38.1 (continued)**  
**General Relationships between Nutrients and Stresses for Turfgrasses**

| Stresses                                     | Relationship between Management Level and Stresses  | Nutrients and Elements That May Have More Significant Impacts and Influences for the Severity of Stresses Than Others | Updated Evidences for Genetic Improvements | References                                                                          |
|----------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------|-------------------------------------------------------------------------------------|
| <b>Turfgrass use and management stresses</b> |                                                     |                                                                                                                       |                                            |                                                                                     |
| Traffic and wear stresses                    | Sports turf and golf course putting greens and tees | N, K, Ca, and Si                                                                                                      | Existed                                    | Trenholm et al. (2001), Hoffman et al. (2010a,b)                                    |
| Cultivations                                 | Golf course putting greens                          | N, P, and K                                                                                                           | Unknown                                    | Fu and Dernoeden (2008), Fu et al. (2009), and Rowland et al. (2009)                |
| Mowing                                       | Golf course putting greens                          | N, P, K, and all nutrients                                                                                            | Unknown                                    | Heckman et al. (2000)                                                               |
| <b>Environmental pollution stresses</b>      |                                                     |                                                                                                                       |                                            |                                                                                     |
| Water pollution and poor water quality       | Arid and semi-arid regions, coastal areas           | N, P, K, and Ca                                                                                                       | Promising                                  | Lockett et al. (2008) and Groffman et al. (2009)                                    |
| Air pollutions                               | Industrial areas                                    | S, N, O <sub>3</sub> , and other greenhouse gases                                                                     | Promising                                  | Groffman et al. (2009)                                                              |
| Soil pollutions                              | Mine industry areas                                 | Heavy metals                                                                                                          | Promising                                  | Qu et al. (2003), Kuo et al. (2005), Yesilonis et al. (2008), and Duo et al. (2009) |

**38.2.1 BIOTIC STRESSES**

Biotic stresses to turf include weeds, diseases, insects and mites, and other living organisms (normally excluding human impacts). Turfgrass nutrients strongly impact on these stresses including influences on turf recovery from these stresses (Liu et al., 2008a,b, 2010, Chapter 39 of this book).

The severity of weed problems is often associated with low maintenance levels. A lower-maintenance-level turf is more vulnerable to weed invasion and proliferation. In general, weeds require less nutrients and water. With improved nutrient-use efficiency, turfgrasses may have a better chance to be able to enhance turf vigor, compete, and even suppress weeds. Turfgrasses compete with weeds for nutrients, and different soil nutrient levels also favor one plant species over another. It is difficult to simply use soil nutrient levels and fertilizers as remedies to control weeds in turf and it will be unlikely a main tool in weed control in the future. However, the difference of nutrient-uptake efficiency between a weed and turfgrass species or cultivar can be further enhanced (Busey, 2003; Turgeon et al., 2009).

Turf diseases have a complicated relationship with mineral nutrients. Excessive nitrogen is in favor of most serious turf diseases in association with soil conditions such as neutral to slightly alkaline soil conditions, which are favorable for some pathogens with the exception of dollar spots and a few other diseases (Liu et al., 2008a,b). Several nutrients, such as Mn and Si (Hill et al., 1999; Datnoff, 2005), have been found to suppress some turf diseases in addition to some mineral elements in pesticides, which is a topic beyond the scope of this chapter. A nutrient-deficient turf will have a difficult time recovering from diseases.

Insects and mites in general like nutritious plants with plenty of protein and amino acid content, and on the other hand, sufficient nutrient supply can help turfgrasses recover from insect and mite damages. Several nutrients or elements such as Si and Al may suppress turf insects and mites by direct unattractive diets or enhanced lignin formation (Potter, 1998).

### 38.2.2 ABIOTIC STRESSES

Nutrients themselves that become deficient or toxic are abiotic stresses to turfgrasses in addition to other abiotic stresses. These stresses include all physically or chemically unfavorable growth conditions (Table 38.1). Proper nutrient supply can minimize stress damage and negative consequences. Under stresses, the unfavorable-condition improvement is the primary step in combination with other approaches including nutrient enhancement that can save energy, costs, and resources. Such approaches require accurate and frequent soil and tissue nutrient analyses.

### 38.2.3 TURF USE AND MANAGEMENT STRESSES

Unlike other yield crop productions in agriculture and horticulture, turfgrasses are maintained for different uses as a functional crop requiring special management practices and skills. Frequent turf mowing is the most unique procedure that sets it apart from any other crops for maintenance requiring specialized mowers and equipment (Beard, 2002; McCarty, 2011). However, lower mowing heights are stressful for turfgrasses during unfavorable growth conditions such as hot summers for cool-season turfgrasses and cooler seasons for warm-season turfgrasses, if mowing with clipping removal consistently depletes turf nutrients by losing nutrients in the cut-off leaves and shoots. In addition, mowing equipment can cause soil compaction and traffic stress to the turf.

In order to improve the turf root system with better oxygen supply and reduced organic matter such as excessive thatch and mat layers, turf cultivation is often applied during the best growing seasons of both cool- and warm-season turfgrasses. Cultivation includes hollow tine core aerification and core removal, solid tine hole aerification, verti-cutting, grooming, hydro-jet, dry-jet, and others. These physical cultivation methods can cause temporary stress to turfgrasses. Proper nutrient supply to supplement the losses by core removal is critical.

Turfgrass traffic, ball marks, divots, and wear are all caused by human use of the turf. Adequate fertilizer input can minimize those stresses to the turfgrass and speed the turf recovery from these stresses.

### 38.2.4 POLLUTION STRESSES

Improper fertilizer input can cause negative consequences to the environment including environmental pollution potentials. Turfgrasses are sinks for pollutants from air, water, and soil, particularly in urban and industrial areas. Proper turfgrass nutrient management can enhance turfgrass's ability to reduce pollution and be a better sink for pollutants. On the other hand, improper and excessive nutrient and pesticide input can cause environmental pollution due to runoff and leaching, etc. Water pollution and poor water quality has been one of the major concerns for turfgrass and environmental relationships. Soil pollution includes mining, which can create toxic levels of some minerals. Turfgrasses can effectively absorb Pb, Cd, Cr, Mn, Ni, Cu, Zn, and other heavy metals to be a part of phytoremediation (Qu et al., 2003; Kuo et al., 2005; Yesilonis et al., 2008; Duo et al., 2009) and function as the first vegetation on oil-shale mined land (Xia, 2004). Turfgrasses are effective absorbers of SO<sub>2</sub>, NO, NO<sub>2</sub>, O<sub>3</sub>, and other greenhouse gases. As the dominant ground cover vegetation in urban areas, those functions are important to protect the environment (Beard and Green, 1994; Carrow et al., 2001; Groffman et al., 2009). In addition, golf courses, sports fields, and sod farms have been sinks for recycling water, biosolid wastes, composts, and sewage wastes (Lockett et al., 2008; Groffman et al., 2009).

As sinks of the above-mentioned pollutants, turfgrass nutrient enhancement can strengthen the turf to overcome these stresses as environmental grasses. The general strategies to reduce these kinds of stresses is to minimize the toxicity and enhance the uptake of deficient nutrients and balance the overall nutrient pool as much as possible, in addition to the proper turfgrass selection suitable for the task. Furthermore, large-scale planning and multiple-dimension approaches using turfgrasses as plant sources for phytoremediation seems needed.

### 38.2.5 DEFINITION OF INTEGRATED STRESS MANAGEMENT

Integrated stress management (ISM) is a strategic approach to control and minimize crop stresses by applying multiple stress relief methods. It has a definition similar to IPM, but ISM includes IPM since a pest problem is a stress to a crop as well. ISM can be applied to any crops or plants that are under stresses, and ISM requires a thorough understanding of stress and the environmental conditions associated with the stress. ISM will be more intensively applied to crops and plants for multiple-stress resistance including nutrient-use efficiency.

ISM can include multiple approaches to control a single stress and multiple approaches to control multiple stresses, with the latter requiring a more ideal maintenance plan for ISM. For example, if a turfgrass can tolerate acidic soil conditions, it has the capability to resist Al toxicity, and it will also have resistance to most patch disease pathogens, which prefer neutral-soil-pH conditions. In addition, by keeping the above condition in favor of the turfgrass, if the turfgrass even has an endophyte infection with natural resistance to insect pests, further enhancement of a turfgrass is a typical ISM approach by direct improvement of Al toxicity resistance and insect resistance, and indirect enhancement of patch disease resistance. Furthermore, the turfgrass may be even more drought resistant than other turfgrasses, which provides new challenges and opportunities for further enhancements. Therefore, ISM also contains endless continuous efforts for crop and plant improvements for stress resistance.

## 38.3 TURFGRASS NUTRITION

Turfgrass nutrition has been well documented in turfgrass textbooks and related literature (Beard, 1973; Carrow et al., 2001; Turgeon, 2008; McCarty, 2011). However, this chapter focuses as a follow-up approach of a recent review of enhancement of nitrogen use under stresses that turfgrasses encounter often during the growing season (Liu et al., 2008a,b).

### 38.3.1 MACRO- AND SECONDARY NUTRIENTS: N, P, K, S, Ca, AND Mg

Turfgrasses require supplemental nutrients from fertilizers. There are six macronutrients, nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg), that are needed in the greatest quantity. Plants contain a concentration range between 0.5% and 6.0% of these six nutrients by dry weight in above-ground tissues. Among these nutrients, N, K, and P are the most critical for turfgrasses (Table 38.2) due to the high potential for deficiency (Carrow et al., 2001).

Nitrogen is the most needed nutrient in quantity for all green plants and crops including turfgrasses. It has been the most actively investigated among all turf nutrients due to N's significant roles for turf growth, color, quality, and use. Nitrogen is a constituent of almost every compound in plants, except carbohydrates, including proteins, chlorophyll, hormones, nucleic acids, and secondary metabolites. Under N deficiency, turf stresses can be worsened and N plays the most significant role among all nutrients to recover turf from these stresses (Liu et al., 2008a,b). On the other hand, excessive N input can increase turf-stress severity, including reduced resistance to extremes of environmental conditions for growth and higher potential damages from pests. Sometimes, excessive

**TABLE 38.2**  
**The 18 Nutrient Elements Required by Higher Plants and Possible Microbial–Host Relationship to Enhance Stress Tolerance**

| Nutrients                                                                                                                                                                         | Chemical Forms Available to Plants                                                                                       | Range of Concentration in Plants (Dry Weight Basis % or ppm) | Main Functions in Plants                                | Potentials for Stress Reduction or Toxicity                                                       | References                                                                                                         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|
| <b>Nutrients that plants obtain from water and air without normal fertilizer input</b>                                                                                            |                                                                                                                          |                                                              |                                                         |                                                                                                   |                                                                                                                    |
| Carbon (C)                                                                                                                                                                        | $\text{CO}_2$ , $\text{CO}_3^{-2}$ , $\text{HCO}_3^-$                                                                    | 40%–45%                                                      | Photosynthesis, carbohydrates                           | Short- and long-term carbon pool                                                                  | Qian and Follett (2002), Qian et al. (2003), and Dai et al. (2009)                                                 |
| Hydrogen (H)                                                                                                                                                                      | $\text{H}^+$ , $\text{H}_2\text{O}$ , $\text{OH}^-$ , $\text{HCO}_3^-$ , and other forms associated with other nutrients | 3%–6%                                                        | Photosynthesis, carbohydrates, pH regulator             | Reduce diseases by providing acidic soil conditions                                               | Hill et al. (1999) and Liu et al. (2008)                                                                           |
| Oxygen (O)                                                                                                                                                                        | $\text{O}_2$ , $\text{CO}_2$ , $\text{OH}^-$ , $\text{H}_2\text{O}$ , and other forms associated with other nutrients    | 40%–45%                                                      | Photosynthesis, carbohydrates, respiration              | Reduce root oxygen stress and waterlogging stress                                                 | Bertrand et al. (2003) and Castonguay et al. (2009)                                                                |
| <b>Macronutrients: primary nutrients with the highest potential for deficiency and required by plants in a range 0.1%–6% in which N is often most needed in adequate quantity</b> |                                                                                                                          |                                                              |                                                         |                                                                                                   |                                                                                                                    |
| Nitrogen (N)                                                                                                                                                                      | $\text{NH}_4^+$ , $\text{NO}_3^-$ , urea, $\text{N}_2$ , amino acids, and other N forms                                  | 2%–6%                                                        | Amino acids, amides, proteins, nucleic acids, coenzymes | Complicated roles and sufficient N will speed recovery from stresses or worsen stresses           | Hull and Liu (2005), Liu et al. (2008), and Baldwin et al. (2009)                                                  |
| Phosphorus (P)                                                                                                                                                                    | $\text{H}_2\text{PO}_4^-$ , $\text{HPO}_4^{2-}$                                                                          | 0.1%–0.6%                                                    | Nucleotides, nucleic acids, phytic acid, actions of ATP | Germination and seedling requirement and association with mycorrhizae to overcome reduced P input |                                                                                                                    |
| Potassium (K)                                                                                                                                                                     | $\text{K}^+$                                                                                                             | 0.5%–4%                                                      | Cofactor of over 40 enzymes, regulator of cell turgor   | Heat, drought, cold, disease, and wear stresses                                                   | Thompson et al. (1995), Miller and Dickens (1996), Trenholm et al. (2001), Cakmak (2005), Hoffman et al. (2010a,b) |

(continued)



TABLE 38.2 (continued)

**The 18 Nutrient Elements Required by Higher Plants and Possible Microbial–Host Relationship to Enhance Stress Tolerance**

| Nutrients                                                                                                                                                                           | Chemical Forms Available to Plants              | Range of Concentration in Plants (Dry Weight Basis % or ppm) | Main Functions in Plants                                                                                                   | Potentials for Stress Reduction or Toxicity | References                                                                                               |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------|--------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|----------------------------------------------------------------------------------------------------------|
| <b>Macronutrients: secondary nutrients with the high potential for deficiency and required by plants in a range 0.1%–2%</b>                                                         |                                                 |                                                              |                                                                                                                            |                                             |                                                                                                          |
| Sulfur (S)                                                                                                                                                                          | SO <sub>4</sub> <sup>2-</sup> , SO <sub>2</sub> | 0.1%–1.5%                                                    | Amino acids including cystine and methionine, coenzyme A, proteins                                                         | Reduce soil pH, black layer                 | Hodges (1992), Berndt and Vargas (2006, 2008)                                                            |
| Calcium (Ca)                                                                                                                                                                        | Ca <sup>2+</sup>                                | 0.1%–6%                                                      | Cell wall lamella, second messenger in metabolism                                                                          | Heat, cold, and stresses                    | Fu and Huang (2003) and St. John et al. (2003)                                                           |
| Magnesium (Mg)                                                                                                                                                                      | Mg <sup>2+</sup>                                | 0.05%–1%                                                     | Chlorophyll, phosphate transfer                                                                                            | Heat, cold, and stresses                    | Kamon (1973), Lee et al. (2007), and Hua et al. (2008)                                                   |
| <b>Micronutrients: nutrients with variable potentials for deficiency and normally required by plants in a range less than 0.1% (1000 ppm); toxicity is found for some nutrients</b> |                                                 |                                                              |                                                                                                                            |                                             |                                                                                                          |
| Iron (Fe)                                                                                                                                                                           | Fe <sup>2+</sup> , Fe <sup>3+</sup>             | 20–600 ppm                                                   | Cytochromes and photosynthesis                                                                                             | Black layer, reduction of P use             | Hodges (1992), Xu and Mancino (2001a,b), and Berndt and Vargas (2006, 2008)                              |
| Manganese (Mn)                                                                                                                                                                      | Mn <sup>2+</sup>                                | 10–600 ppm                                                   | Dehydrogenases, photosynthesis, and O <sub>2</sub> evolution                                                               | Disease reduction                           | Hill et al. (1999)                                                                                       |
| Zinc (Zn)                                                                                                                                                                           | Zn <sup>2+</sup>                                | 10–250 ppm                                                   | Alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase                                                          |                                             | Hull (2001), Xu and Mancino (2001a,b), and Hua et al. (2008)                                             |
| Copper (Cu)                                                                                                                                                                         | Cu <sup>2+</sup>                                | 1–20 ppm                                                     | Conversion of amino acids to proteins, the formation of carbohydrates during photosynthesis and in the formation of lignin | Disease reduction                           | Curvetto and Rauser (1979), Hill et al. (1999), Faust and Christians (1999, 2000), and Hua et al. (2008) |
| Boron (B)                                                                                                                                                                           | H <sub>3</sub> BO <sub>3</sub>                  | 0.2–800 ppm                                                  | Cell elongation and nucleic acid metabolism                                                                                |                                             | Guertal (2004)                                                                                           |

**TABLE 38.2 (continued)**  
**The 18 Nutrient Elements Required by Higher Plants and Possible Microbial–Host Relationship to Enhance Stress Tolerance**

| Nutrients       | Chemical Forms Available to Plants      | Range of Concentration in Plants (Dry Weight Basis % or ppm) | Main Functions in Plants                               | Potentials for Stress Reduction or Toxicity | References                                    |
|-----------------|-----------------------------------------|--------------------------------------------------------------|--------------------------------------------------------|---------------------------------------------|-----------------------------------------------|
| Molybdenum (Mo) | $\text{MoO}_4^{2-}$ , $\text{HMoO}_4^-$ | 0.1–10 ppm                                                   | Nitrogenase, nitrate reductase, xanthine dehydrogenase | Enhancement of symbiotic relationship       | Gupta (1997) and Kaiser et al. (2005)         |
| Chlorine (Cl)   | $\text{Cl}^-$                           | 10–80,000 ppm                                                | Photosynthesis and $\text{O}_2$ evolution              | Disease control                             | Thompson et al. (1995) and Mann et al. (2004) |
| Nickel (Ni)     | $\text{Ni}^{2+}$                        | 0.05–5 ppm                                                   | Urease                                                 | Foliar N absorption                         | Chen et al. (2009)                            |

**Beneficial nutrients: nutrients required by some plants, and except Si, the concentration range of which in plants is similar to micronutrients. Toxicity is found for some nutrients**

|              |                                                              |                 |                                                                                                 |                                                                             |                                                                                                                                         |
|--------------|--------------------------------------------------------------|-----------------|-------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|
| Silicon (Si) | $\text{H}_2\text{SiO}_4$                                     | 0.1–100,000 ppm | Cell wall rigidity and elasticity                                                               | Disease, insect, wear, heat resistance, reduction of other element toxicity | Street et al. (1981), Trenholm et al. (2001), Datnoff et al. (2007), Brecht et al. Liang et al. (2007, 2009), and Vaculka et al. (2009) |
| Sodium (Na)  | $\text{Na}^+$                                                | 10–80,000 ppm   | Regeneration of phosphoenolpyruvate in $\text{C}_4$ and CAM plants, substitute for $\text{K}^+$ | Disease, weed control, nutrient imbalance, cold tolerance                   | Munshaw et al. (2004)                                                                                                                   |
| Selenium     | $\text{Se}^{2-}$ , $\text{SeO}_3^{2-}$ , $\text{SeO}_4^{2-}$ | 0.1–25 ppm      | Selenoproteins, selenoenzymes                                                                   | Disease resistance                                                          | Hopper and Parker (1999), Li et al. (2008), and Zhu et al. (2009)                                                                       |

| Microbes                          | Nutrients Benefited | Type of Association with Host Plants | Main Benefits to Host Plants                 | Potentials for Stress Reduction                | References                                                                                                                                         |
|-----------------------------------|---------------------|--------------------------------------|----------------------------------------------|------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Neotyphodium</i><br>Endophytes | P, others           | Endo infection above-ground tissues  | Insect pest resistance and stress resistance | Al tolerance, drought stress, P-use efficiency | Funk et al. (1983), Latch (1993), Liu et al. (1996), Malinowski and Belesky (1999, 2000), Johnson-Cicalese et al. (2000), and Zaurov et al. (2001) |

(continued)

TABLE 38.2 (continued)

**The 18 Nutrient Elements Required by Higher Plants and Possible Microbial–Host Relationship to Enhance Stress Tolerance**

| Microbes                              | Nutrients Benefited | Type of Association with Host Plants                          | Main Benefits to Host Plants                                                                             | Potentials for Stress Reduction       | References                                                                                                              |
|---------------------------------------|---------------------|---------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------------------------------------------|
| Arbuscular-mycorrhizal (AM) symbiosis | P, N, others        | Roots                                                         | P, water, and other nutrient uptake and stress resistance                                                | Drought tolerance                     | Subramanian and Charest (1995), and Pelletier and Dionne (2004)                                                         |
| Root zone and rhizosphere bacteria    | N and C             | Non-pathogenic and with habitats in root zone and rhizosphere | Reduction of excessive thatch and organic matter in root zones, enhancement of abiotic stress resistance | Drought and salinity stress reduction | Figueiredo et al. (2008) and Yang et al. (2009)                                                                         |
| Rhizobia, diazotrophic bacteria       | N <sub>2</sub>      | Root nodules                                                  | Nitrogen fixation                                                                                        | Promising                             | Raimam et al. (2007), Bi et al. (2009), Bonfante and Anca (2009), Heath and Tiffin (2009), and Prakamhang et al. (2009) |

N may be even worse than slight N deficiency to manage turf under stresses regardless of N loss potential from the turf-soil system.

Potassium is the second highest concentrated nutrient in tissue in both cool-season and warm-season turfgrasses with a range of 1%–4%. Unlike most nutrients, K does not form any compounds in plants but it plays significant roles in plant metabolism as a monovalent cation. Potassium is required by more than 40 enzymes as a cofactor to function in plants (Marschner, 1995). Potassium is the principal cation to establish cell turgor and regulates plant hormones associated with water use, stress resistance, and nutrient balance in plants. Potassium deficiency is often found in sandy soils and it competes with other cations for plant absorption and soil CEC sites. Negative impacts of K deficiency to turf stresses is much more significant than the excessiveness of K for turfgrasses. Potassium toxicity is rarely reported for turfgrasses. According to a recent survey, K input to golf course greens already surpassed N input in the United States (Throssell et al., 2009). In the future, K may be listed second next to N as NKP, instead of NPK (a very important order for crop yield production) according to the deficient potential particularly associated with sandy soils for turf growth even with relatively high organic matter content (Carrow et al., 2001).

Phosphorus concentration in turfgrass tissues ranges from 0.2% to 0.6% by dry weight and P is a component of ATP, sugar phosphate, nucleic acids, phospholipids, phytic acids, and coenzymes. Phosphorus plays more significant roles in turf seedlings than mature turf in both cool-season and warm-season turfgrasses (Carrow et al., 2001). P is very often deficient due to its low concentrations (<10 mg L<sup>-1</sup>) in most soil solutions. In acidic soils, high soluble aluminum and iron can react with P and form insoluble phosphate salts to cause severe P deficiency and metal toxicity. Excessive

P applications can cause runoff, leaching, and contamination of P to the environment and water sources (Carrow et al., 2001; Sims and Sharpley, 2005; Soldat and Petrovic, 2008).

Sulfur is required by plants to synthesize the S-containing amino acids cystine, cysteine, and methionine. Grasses normally contain 0.1%–0.5% S by dry weight. Sulfur deficiency is often observed in salt-affected sandy soils with high pH. Frequent removal of clippings can cause S depletion from some soils. However, frequent use of sulfur-coated urea and sulfate-containing fertilizers, including gypsum ( $\text{CaSO}_4$ ), can add S to turfgrass–soil systems. Acidic soil conditions and high S contents in soils can suppress some patch diseases such as take-all patch, summer patch, and spring dead spot (Dernoeden and O’Neil, 1983; Carrow et al., 2001; Vargas, 2005). Sulfur can be found under anaerobic conditions associated with excessive Fe to form black layers in soils (Hodges, 1992; Carrow et al., 2001; Vargas, 2005). Black layers often occur in anaerobic soil conditions created by compaction and/or excessive irrigation. The anaerobic conditions cause sulfur-reducing bacteria to use sulfate or elemental sulfur instead of oxygen during the respiration process, which produces hydrogen sulfide ( $\text{H}_2\text{S}$ ) gas, a poisonous compound to turfgrass roots. The hydrogen sulfide reacts with metal elements such as Fe, creating black deposits of  $\text{FeS}$ , which form black layers within the soil and inhibit turfgrass growth. The use of sulfuric acid to treat irrigation water is a common practice where carbonate ( $\text{CO}_3^{2-}$ ) and bicarbonate ( $\text{HCO}_3^-$ ) levels are high and excess sodium (Na) exists. Bicarbonates can react with Ca and Mg in the water and can remove these cations from solution. This reduction of soluble Ca and Mg results in an increase in the sodium adsorption ratio (SAR) of the water, which increases the likelihood of soil structure problems caused by Na deflocculating clay particles (Christians, 1999). The process of adding sulfuric acid to irrigation water requires a thorough evaluation of the soil conditions and water chemistry before it is initiated.

Calcium primarily is passively absorbed by plants at the root tip as  $\text{Ca}^{2+}$ . The concentration of Ca found in grasses range from 0.3% to 1.25% of dry matter (Carrow et al., 2001), which varies among turfgrass species and cultivars. Normally, irrigated turfgrass can receive appreciable amounts of Ca from the irrigation water. Calcium plays important roles that affect the susceptibility to stresses in four ways. First, Ca is important for the stability and function of plant membranes. When there is Ca deficiency, membrane leakage occurs with low-molecular-weight compounds, e.g., sugars and amino acids, from the cytoplasm to the apoplast, which may stimulate infection by some pathogens (Marschner, 1995). Second, Ca is an important component of the cell wall structure required in the middle lamella for stability. When Ca concentration drops, there is an increased susceptibility to stress, causing wilting symptoms. Third, Ca and K work together to activate ABA in stomata closure and opening in responding to water deficit of cells. Fourth, Ca is a secondary messenger in plants and it involves signaling messages for heat, drought, and lower temperature stresses. In addition, Ca may be involved for seed production of crops to protect from diseases and stresses. For turfgrass management, Ca deficiency and toxicity are less frequently observed than the incidences of nutrient imbalances of Ca associated with other cations (Carrow et al., 2001).

Magnesium is absorbed from the soil as the  $\text{Mg}^{2+}$  ion. The Mg requirement for plant growth ranges from 0.15% to 1% of the dry weight. Magnesium is essential for a number of fundamental biochemical processes in all living cells. For example,  $\text{Mg}^{2+}$  ions are involved in the interaction of the ribosome subunits, the counter-ions of ATP, and the central ions in chlorophylls. Magnesium is a cofactor in numerous enzymes mainly involved in nucleotide metabolism (Marschner, 1995). Less than 0.15% dry leaf tissue Mg content is usually considered deficient and will vary depending upon species and cultivar (Carrow et al., 2001). Turfgrass Mg deficiencies commonly occur in acidic soils and soils that receive high application rates of basic cations. Saline or effluent waters with high concentrations of Ca, K, or Na can be negative for the soil Mg availability and Mg uptake. Sandy soils with low CEC receiving high irrigation rates are also susceptible to Mg loss through leaching. The direct involvement of magnesium in stress relief in turfgrasses has not been reported, but magnesium’s essential role in photosynthesis helps turfgrasses in carbohydrate production and to overcome stress, particularly after cultivation, during the most active growing seasons of both cool-season and warm-season turfgrasses.

### 38.3.2 MICRONUTRIENTS: Fe, Cu, Zn, Mn, Cl, B, Mo, AND Ni

Iron has received the greatest attention of all the micronutrients, because of its effects on turfgrass appearance, color, and stress management (Carrow et al., 2001; Liu et al., Chapter 39 of this book). For turfgrasses, micronutrient deficiencies do not always show clear symptoms due to relatively fine leaf textures and non yield production in comparison with other crops. Actual deficiencies of Cu, Zn, Mn, B, Cl, Mo, and Ni are rarely reported in turfgrasses, but toxicities of Cu, Zn, Mn, Cl, B, Mo, and Ni can occur in areas contaminated by mining or other industry operations. Soil amendments with high heavy metal contents or excessive use of micronutrient fertilizers can cause turfgrass stresses. With proper input or at normal ranges of concentrations, the most significant roles of micronutrients are to reduce plant stresses in addition to their essential physiological and biochemical functions to plants. Their direct and indirect roles in pest resistance and control, particularly with diseases, can be grouped as (1) pests are sensitive to some micronutrients, (2) some micronutrients are constituents of pesticides, (3) some micronutrients can function as protective or curative agents for host plants physically or chemically, and (4) some micronutrients can have indirect impacts on pest by forming symbiotic relationships with other microbes. In addition, certain ratios of micronutrients and their interaction with each other can have impacts to plant stresses (Dordas, 2008).

### 38.3.3 BENEFICIAL NUTRIENTS: Si, Na, AND Se

Silicon has been most actively studied in recent years as a beneficial nutrient for plants including turfgrasses, and numerous reports have identified its positive roles in stress relief and control and pest management. Evidence shows three major aspects of silicon's roles in plant protection and stress relief in addition to its physiological roles being identified in crops such as rice. First, Si may form a physical protection barrier as silicate on plant leaf surfaces between the cuticle layer and epidermal cells. Second, Si stimulates antioxidant systems in plants. Third, Si interacts with other toxic metals to reduce toxicity (Liang et al., 2007).

Sodium is not an essential plant nutrient, and for cool-season turfgrasses, excessive Na is toxic to  $C_3$  plants. However, Na is an essential-nutrient to warm-season turfgrasses, particularly for salinity tolerant species, which deserve more attention (Munshaw et al., 2004). Sodium also plays a positive role in protection from freezing damages and suppression of weeds and pests. Although Na has never been reported being deficient for turfgrasses, it is often found that turfgrass tissue dry weight contains about 10–100 mg kg<sup>-1</sup> of Na when turfgrasses are grown in sandy soils without salinity stress.

Selenium is an essential micronutrient for many organisms, including plants, animals, and humans, with a function chemically similar to sulfur. As a plant nutrient, Se ranges are 0.1–25 ppm by dry weight in plants, and its main function is to form selenoproteins and selenoenzymes, which have protective functions from stresses. Although Se functions in plants have not been fully identified, Se is normally taken up by plants as selenate,  $\text{SeO}_4^{2-}$ , and Se is rarely reported deficient. Most Se research focuses on its toxicity to plants and in soils particularly for biofortification and phytoremediation of Se-contaminated environments since some crops reported have the capability to accumulate Se (Hopper and Parker, 1999; Li et al., 2008; Zhu et al., 2009). To the authors' knowledge, Se deficiency and toxicity of turfgrasses have not been reported. In addition to the three beneficial nutrients, others have been reported and will not be discussed in this chapter.

### 38.3.4 MICROBIAL–HOST RELATIONSHIPS

The general interactions of plant mineral nutrients and soil microbes are not the focus of this chapter, but several relationships are important for turfgrass nutrient management under stresses related to nutrient use directly or indirectly.

Grasses infected with *Neotyphodium* spp. endophytes have an extraordinary impact on the ecology and economy of turfgrasses (Funk et al., 1983, 1994). Endophytes induce drought resistance and mineral nutrient (N, P, Ca) use efficiency, which affect production of ergot alkaloids, which are toxic to livestock living on forage crops, but are beneficial for turfgrasses, offering protection from insect pest attacks. It was reported that endophyte-infected tall fescue had an enhanced P uptake under P deficiency stress. The mechanisms were altered root morphology with reduced root diameters and longer root hairs and chemical modification of the rhizosphere resulting from exudation of phenolic-like compounds, which can form Al-chelating compounds in the rhizosphere to protect hosts from Al toxicity (Funk et al., 1983; Latch, 1993; Liu et al., 1996; Malinowski and Belesky, 1999, 2000; Johnson-Cicalese et al., 2000; Zaurov et al., 2001).

Pelletier and Dionne (2004) reported that two mycorrhizal species, *Glomus intraradices* Schenck & Smith and *G. etunicatum* Becker & Gerdemann, affected the establishment of a lawn mixture of Kentucky bluegrass (*Poa pratensis* L.), red fescue (*Festuca rubra* L.), and perennial ryegrass (*Lolium perenne* L.). Turfgrass inoculated with *G. intraradices* at rates between 40 and 60 mL m<sup>-2</sup> established more quickly than turfgrass inoculated with *G. etunicatum* when inoculated at time of seeding, with no irrigation or fertilization inputs. These results confer arbuscular-mycorrhizal (AM) symbiosis' benefits to host plants, including improved tolerance to abiotic and biotic stresses. Although the majority of turfgrasses form an AM symbiosis in most soils, little is known of the mycorrhization of turfgrass species for stress control.

Plant-growth-promoting rhizobacteria (PGPR) are associated with plant roots and the thatch layer of turfgrasses. Recent work by several groups shows that PGPR may have functions for salinity and drought tolerance enhancement (Figueiredo et al., 2008; Yang et al., 2009). Although it has not been reported for turfgrasses, PGPR may have effects to reduce the need for fertilizers and prevent the accumulation of nitrates (denitrifiers), sulfate, and phosphates (S and P efficient users) in turf soils.

Unlike legumes, grasses cannot fix nitrogen directly from N<sub>2</sub> as the major N supply, but some grasses including rice and maize as the major crops can fix certain amounts of nitrogen through different types of rhizobacteria, and the future is promising as far as application to turfgrasses is concerned (Raimam et al., 2007; Bi et al., 2009; Bonfante and Anca, 2009; Heath and Tiffin, 2009; Prakamhang et al., 2009) to further reduce nitrogen input to turf areas.

## 38.4 MAJOR MANAGEMENT STRATEGIES TO ENHANCE TURFGRASS NUTRIENT USE UNDER STRESSES

### 38.4.1 MOWING

Mowing removes foliar parts and shoots of turfgrasses, and in general lower mowing heights have negative impacts to turf stresses except for physical removal of weeds and turf shoot density enhancement. However, in order to meet the turf use and function, low mowing heights for turf lower than 2.5 mm for golf putting greens are required. The major strategies to enhance nutrient use for a lower mowing height situation include (1) frequent and light fertilizer applications during the growing season; (2) increased utilization of liquid and foliar fertilizers than granular fertilizers; (3) recycling clippings to regain nutrients from clippings when it is allowed to maintain the turf use and quality; (4) frequent mowing to avoid excessive removal of clippings at each single mowing event; (5) use of lower nutrient demand or nutrient-use efficient turfgrass species or cultivars; (6) use of turf paint to maintain turf color to avoid winter overseeding, fertilizing, and mowing; (7) use of plant growth regulators (PGR) to reduce growth and nutrient input without compromising turf quality; (8) use of turfgrass species and cultivars, which can tolerate lower mowing heights; and (9) use of turfgrass species and cultivars with deeper root systems and better capability to take up nutrients to minimize potential of nutrient losses.

Lower and more frequent mowing with clipping removal increases turfgrass nutrient demand and is why golf course putting greens require more fertilizer input than other turf areas during

the growing season. Raising the mowing height will help turfgrass nutrient use under stress. The relationship between mowing and turf stress has been thoroughly and intensively investigated (Dernoeden, 2000; Beard, 2002; McCarty and Miller, 2002; McCarty, 2011). Putting greens in a range of 2.5–5 mm have received the most attention and need the most improvement in nutrient-use enhancement and ISM to overcome stresses. It is never overemphasized that slightly raising the mowing height of a putting green as allowed can be the key factor in stress relief, particularly during the unfavorable growing conditions for both cool- and warm-season turfgrasses.

Reduced mowing height, associated with plant growth regulator application on turf, can enhance turf nutrient-use efficiency and even reduce fertilizer input without compromising turf quality and function. Lower mowing heights associated with proper water management and other management practices can also enhance turf nutrient-use efficiency and overcome turf stresses.

### 38.4.2 IRRIGATION

Proper irrigation can enhance turfgrass nutrient use under stressful conditions. The general strategies may include two main aspects (1) the irrigation practice itself and (2) water sources applied to turfgrasses. Under stressful conditions, moistening but not waterlogging the root zone will assure water and nutrient requirements of turfgrass are met.

The turf irrigation practices to enhance nutrient use under stresses may include several general approaches: (1) watering turf as needed and avoiding excessive irrigation; (2) avoiding scheduled irrigation by using a sensing system to understand the exact turf water need; (3) watering the turf in the early mornings to reduce stress; (4) watering only at the most needed area to avoid broadcasting irrigation; (5) using wetting agents and PGR to save water and minimize stress; (6) selecting proper irrigation systems suitable for the turf, without or with only minimized negative impacts to stresses; and (7) using a flexible fertigation system and effluent water. Irrigation design should be adapted to the site specific for turf use and playability to avoid uneven irrigation causing or worsen stresses.

Poor water quality will worsen turf stresses. Using poor-water-quality-tolerant turfgrasses can minimize the water quality problem impact to turf stresses in addition to the correction of the poor water quality. Pure, potable water used for irrigation has minimum influence on available nutrient concentrations in the soil. However, poor water and effluent water can change nutrient concentrations and soil chemistry, leading to negative and even detrimental impacts to turfgrasses. Imbalanced nutrient supply and element toxicity are often encountered by using poor quality water and effluent water. Frequent soil nutrient and turfgrass tissue tests, and soil management can provide guidelines to correct poor quality water stress and enhance nutrient use to focusing on other stresses.

### 38.4.3 FERTILIZATION

Nutrient deficiency and toxicity cause stress on turf. These stresses, in conjunction with other abiotic and biotic stresses, can be complicated problems to solve, since there are more than 18 elements that a turfgrass needs. Each nutrient must be in the proper range of supply and interactions among the 18 elements exist consistently. The concept of “apply the exact fertilizer as the turf needs” makes perfect logical sense, but it is difficult to practice due to the unique aspects of turfgrass management that lack a uniform measurement parameter for quality, such as yield. The evaluation of turf quality and appearance can be subjective, depending on location, turfgrass species, turf use, climatic condition, and individual evaluator. The following reasons may contribute to the complex nature of fertilization management for turf under stresses: (1) turfgrass management is not for production and it is harder to measure the exact need for fertilizers; (2) the primary nutrients, NPK, do not play a significant role here as with yield production crops do; (3) greater differences in nutrient requirement exist among turfgrass species, cultivars, and turf use; (4) unique maintenance procedures are highly associated with

nutrient status such as clipping removal from turf; and (5) as perennial functional crops, turfgrasses have thatch layers and receive traffic and soil compaction stresses, which are highly associated with nutrient-use efficiency.

For professional turfgrass management, frequent soil and tissue testing with accurate interpretation of the turfgrass nutrient status under stresses may be the most important first step before carrying out any fertilization practices.

#### 38.4.4 PEST MANAGEMENT

The turf pests include weeds, diseases, insects and mites, and others, which either use turfgrasses as their food source or habitat on turfgrasses, causing physical damages or aesthetic appearance disturbance. Enhancing turfgrass nutrient use under these pest stresses is part of IPM, requiring a thorough understanding of turfgrass nutrient needs and pest control methods. The general strategies may include the following.

First, an understanding of the major or dominant nutrient elements associated with the current or potential pest problem is required. A lower soil pH will have a positive function, suppressing patch disease pathogens such as take-all patch, brown patch, and spring dead spot, which are very serious diseases for creeping bent grass and hybrid Bermuda grasses. With a lower-soil-pH approach, other potential impacts to turfgrass nutrients must be considered such as reduced availability of soil P and increased soluble Fe, Al, and Mn. Soil Mn has a positive effect to suppress take-all patch. Unlike the other two basic turfgrass practices of watering and mowing, turfgrass fertilizer addition involves the addition of over 18 nutrient elements, and each nutrient has its unique way of affecting turf quality and pest potential. The 18 turfgrass nutrients interact with each other, and it is often a combination of effects that work out positively or negatively to turfgrasses.

Second, an understanding of nutrient ratios and nutrient interaction impacts to pest problems and potential is required. During new turfgrass establishment, P input is encouraged. However, weed species such as carpetweed and other common summer annuals are favored by a high P supply and compete with the warm-season turfgrasses during establishment.

Third, enhancing turfgrass with microbial association including endophytes and mycorrhizae, and rhizosphere microbial activities would be beneficial. These associations benefit turfgrasses. For example, mycorrhizae can enhance turfgrass P uptake and suppress Fe deficiency. Endophyte infected turfgrasses have a better Al toxicity tolerance (Table 38.2).

ISM includes IPM since pests are biotic stresses, and research is needed to enhance ISM related to turfgrass nutrients.

#### 38.4.5 ROOT ZONE MANAGEMENT

Turfgrasses belong to shallow rooted crops and all grasses have fibrous root systems. Root zone management of turfgrass includes cultivation, thatch control, root growth enhancement, soil modification and amendment, edaphic problem correction, and edaphic environment enhancement.

Turfgrass root zone management with modified sandy root zones either with USGA green (85/15 v/v of sand and peat moss) or California green (100% sand) are commonly used for golf putting greens and sports fields. These modified root zones significantly improve water percolation and also face the challenge of low nutrient retention in the root zone.

The most significant turfgrass management practice that disturbs a golf course's playability is core cultivation. Speedy recovery from core cultivation requires proper nutrient supply. Other management techniques, such as sub-air system for altering soil CO<sub>2</sub>, O<sub>2</sub>, soil solution levels, and microbial activities, require research regarding abiotic and biotic stresses.

Fu et al. (2009) reported that both spring-only and especially spring-plus-summer coring caused substantial reductions in turf quality for a 2-week period. Spring-plus-summer coring resulted in increased chlorophyll levels as well as improved turf color and quality in late summer of 'providence'



creeping bent grass putting green. During the two-year study period in 2006 and 2007, the bent grass was fertilized biweekly with  $4.9 \text{ kg N ha}^{-1}$  of urea between May 1 and June 7, and then weekly through August 24, for a total of  $78.4 \text{ kg N ha}^{-1}$ , during the experimental period in 2006. In the autumn of 2006,  $71 \text{ kg N ha}^{-1}$  was applied between September and November. In 2007, the bent grass was fertilized weekly at a rate of  $4.9 \text{ kg N ha}^{-1}$  with urea, between April 30 and August 27, to provide a total of  $88.2 \text{ kg N ha}^{-1}$  during the experimental period. Although fertilizer rates were not compared for the study, the lower rates and frequent application of urea during summer months were important for the recovery from cultivation stresses.

#### 38.4.6 PLANT GROWTH REGULATORS, SURFACTANTS (WETTING AGENTS), AND BIO-STIMULANTS

For fine turfgrass management, such as in golf course putting greens, tees, fairways, and sports fields, PGR, wetting agents, and bio-stimulants are commonly used. These products have been applied to turfgrass in the past several decades and the use of these products will likely continue to grow. The major benefits of these products to turfgrasses include reduction of drought, heat, shade, and other stresses, in addition to reduced mowing frequency and enhanced turf appearance. The association of these products with turfgrass nutrient and nutrient-use efficiency needs more attention.

The combination of trinexapac-ethyl and reduced N input improves both cool-season and warm-season turfgrasses (McCullough et al., 2006; Baldwin et al., 2009). Summer applications of turf surfactants plus K, Ca, and Mg input improve creeping bent grass summer stress (Fu and Huang, 2003; Sarvis, 2008). Bio-stimulants enhance turfgrass stresses to shade, drought, heat, and cold (Zhang et al., 2010).

Application of cytokinins has been shown to increase endogenous cytokinin content to improve plant stress tolerance. Goatley and Schmidt (1990) found that foliar application of 6-benzyladenine (6-BA) delayed senescence of Kentucky bluegrass (*P. pratensis* L.). Liu et al. (2002) reported that rootzone injection of zeatin riboside increased endogenous cytokinins and antioxidant activity and delayed senescence in heat-stressed creeping bent grass. Wang et al. (2006) showed that the synthetic cytokinin, 6-BA, at  $50 \mu\text{g L}^{-1}$  also increased creeping bent grass heat resistance. Recently, Zhang et al. (2010) reported that seaweed (*Ascophyllum nodosum* Jol.) extract-based cytokinins (SWEC) led to improvements in turfgrass stress tolerance. Repeated foliar application of SWEC at  $10 \mu\text{M}$  may be an effective approach for improving turfgrass performance under heat stress. Increased heat stress tolerance and repeated applications of SWEC might have an enhancement of N use in creeping bent grass.

### 38.5 TURFGRASS IMPROVEMENT TO ENHANCE NUTRIENT USE UNDER STRESSES

#### 38.5.1 IMPROVED NUTRIENT UPTAKE AND USE

Differences in nutrient-uptake kinetics and enzyme activities exist in cool-season turfgrasses, leading to different nutrient-use efficiency (Cisar et al., 1989; Jiang and Hull, 1998; Bushoven and Hull, 2001, 2005; Hull and Liu, 2005; Liu and Hull, 2006). These differences can be further enhanced for future turfgrass improvement related to nutrient use, particularly under stresses. Guertal and Hicks (2009) reported that N source treatments of ammonium nitrate (AN) (34-0-0) or calcium nitrate (16-0-0; 39% Ca), both applied at 2.4, 4.9, 7.3, or  $9.8 \text{ g N m}^{-2} \text{ week}^{-1}$  to “TifSport” and “Tifway” Bermuda grasses for 2 years resulted in TifSport having a higher percentage of groundcover than Tifway. Shoot density of both turfgrasses increased as N rate increased, with shoot density maximized at N rates between 6.2 and  $7.6 \text{ g N m}^{-2} \text{ week}^{-1}$ . In both years 2003 and 2004, it was found that dry weight of stolons and rhizomes decreased as N rate increased. Guertal and Hicks (2009) also found that the highest rate of N used in the study ( $9.8 \text{ g N m}^{-2} \text{ week}^{-1}$ ) was not needed for

satisfactory growth of hybrid Bermuda grass. Lower rates of either  $\text{NH}_4\text{NO}_3$  or  $\text{Ca}(\text{NO}_3)_2$  fostered turfgrass coverage without subsequent reduction in the dry weight of important turfgrass stems (stolons and rhizomes). Nitrogen source rarely affects the percentage of turf groundcover, shoot density, or dry weight of stolons and rhizomes, which was found by Stiglbauer et al. (2009) in “Diamond” zoysiagrass establishment. These findings indicate genetic differences exist for both cool-season and warm-season turfgrasses in nutrient use, and the future of turfgrass nutrient-use improvement is promising.

### 38.5.2 STRONGER COMPETITIVENESS AND FITNESS

Garrison and Stier (2010) studied 10 turfgrass species planted into two anthropogenic prairies and monitored over a 2 year period. They found that colonies of most species, including Kentucky bluegrass (*P. pratensis* L.), creeping bent grass (*Agrostis stolonifera* L.), and tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort = *Lolium arundinaceum* (Schreb.) Darbysh, formerly *Festuca arundinacea* Schreb. var. *arundinacea*], decreased in size or died due to herbivores and environmental stresses. However, at one location, fine fescues (*Festuca* spp.) and colonial bent grass (*A. capillaris* L.) colonies remained relatively constant or slightly increased in size, while native velvet bent grass (*A. canina* L.) nearly tripled in colony diameter.

As mono-cultured crops, turfgrasses are rarely mixed with other grasses. The new cultivars of turfgrass species must have a high affinity transporter (HAT) for nutrient uptake, allowing turfgrasses to be able to compete with weeds when external soil solutions with lower nutrient concentrations. HATs would require less active (energy cost) uptake leading to increased uptake efficiency under nutrient-deficient stress (Hull and Liu, 2005; Liu et al., 2008a,b).

### 38.5.3 MODIFIED GROWTH HABIT AND APPEARANCE

Turfgrasses can be genetically modified in growth habits and appearance to meet turf needs such as finer leaf textures, enhanced horizontal growth habits with more stolons and rhizomes, and dwarf types to tolerate lower mowing heights. Such changes may or may not enhance stress tolerance but proper nutrient management will benefit these modified turfgrasses to overcome stress.

Genetically modified turfgrasses should have larger roots rather than smaller roots to overcome stressful conditions. Li et al. reported (2010) that the *Arabidopsis* vacuolar  $\text{H}^+$ -pyrophosphatase (AVP1), when over-expressed in creeping bent grass transgenic (TG) plants, regulated root and shoot development via facilitation of auxin flux and enhanced plant resistance to salt and drought stresses. TG plants exhibited greater biomass production than wild type (WT) controls under both normal and elevated salinity conditions. When subjected to salt stress, fresh (FW) and dry weights (DW) of both leaves and roots decreased more significantly in WT compared to TG plants. These results demonstrate the great potential of genetic manipulation of vacuolar  $\text{H}^+$ -pyrophosphatase expression in TG perennial species, for improvement of plant abiotic stress resistance with greater root growth.

Rhizomatous growth habit is the preferred horizontal growth that helps turfgrass to form an even ground cover. Tall fescues (Jernstedt and Bouton, 1985; De Battista and Bouton, 1990) have been identified and investigated for potential enhancement in rhizomatous growth habit. In addition to the morphological roles of turfgrass rhizomes, nutrient related roles of rhizomes seem leading to opportunities for enhancement in practices. Kavanová and Gloser (2005) studied the nitrogen stores in a rhizomatous perennial grass (*Calamagrostis epigejos*) to investigate regrowth after defoliation. They found that roots were the main net source of mobilized N, and the root's dominant N storage compounds were free amino acids. Free amino acids and soluble proteins in the roots decreased by 55% and 50%, respectively, and a substantial (38%) decrease in stubble protein was also observed after defoliation. Although the relative abundance of several soluble proteins in roots decreased during the initial recovery from defoliation, no evidence was found for vegetative storage protein

(different from free amino acids and proteins) in rhizomes. They concluded that new leaf regrowth was entirely reliant on N stores present in the plant roots after defoliation. Mobilized N originated mainly from free amino acids and soluble proteins located in roots. Their data suggest that rhizomes play an important role in N transport but not in N storage. Such a finding may be important for winter overseeding practices, related to N and other nutrients. Before overseeding, an aggressive vertical mowing can damage more rhizomes than roots for a warm-season turfgrass. This damage could result in the lost function of rhizomes for the next spring transporting function of N and other nutrients from roots to new leaves when green up starts.

#### **38.5.4 INTEGRATED MANAGEMENT SCALES TO ENHANCE SUSTAINABILITY**

With financial challenges and competition for golfers in the golf industry worldwide, new strategies must be implemented to enhance the management skills and economics of the turf industry to prepare for the future. Labor costs, energy costs, resource costs, and other costs can be creatively reduced by more integrated cooperation between golf courses beyond individual courses despite ownership and management structure. Recycling resources used for turfgrasses are also important for future turfgrass management. For turfgrass nutrient management, the recommendation of nutrient use is not only based on certain species or cultivars used, but also on certain geographical locations and climates used with specific mowing heights and water use standards. Site-specific nutrient sensing and application will further enhance nutrient-use efficiency (Xiong et al., 2007; Bell and Xiong, 2008; Krum et al., 2010). Finally, the discrepancy between essential management requirement and public perceptions for turfgrass management input will need to be further corrected and minimized by large-scale research (Groffman et al., 2009), extensive outreach, and public educational programs.

#### **38.5.5 FUTURE TURFGRASS FERTILIZERS AND APPLICATIONS**

It is unlikely that the costs for turfgrass fertilizers will go down because of several reasons: (1) costs of energy, (2) costs of labor, (3) more limited resources to produce fertilizers, (4) more expensive costs of fertilizer transportation, and (5) increased regulations implemented in fertilizer production.

The future turfgrass fertilizers may be required to meet the following to be (1) more responsive to turfgrass soil and tissue test results; (2) increased liquid fertilizer production and application; (3) new formulations with pesticides, surfactants, bio-stimulants, colorants, and others; (4) increased production of natural and organic fertilizers with new technologies being implemented; (5) more microbial activity engaging and enhancing fertilizers; and (6) more turfgrass fertilizers designed to be applied to turf under stresses to speed the recovery and minimize stresses.

### **38.6 SUMMARY AND PROSPECTS**

Turfgrass fertilization faces new challenges, demanding new and exciting technologies and management strategies. Limited resources and restricted regulations can positively contribute to a reduction of excessive input potential of turf fertilizers, but sound fertilizer management programs, as a part of ISM, is critical to maintain turfgrasses healthy and functional in nutrient aspects and benefit the environment. As functional crops, the economic, environmental, social, and political values of turfgrasses vary in different societies and locations. In spite of those differences, stressed turfgrasses are often seen and maintained as they are, compared to yield producing crops. However, it is unnecessary to reach the maximum growth rate for turfgrasses to be the best functional crops and it is often that slightly deficient turfgrass nutrients are recommended.

Over the past 50 years, most studies have focused on complex turfgrass nutrition problems associated with N, P, K, S, Ca, Mg, Fe, and Si, and have received the greatest attention because of their effects on turfgrass appearance, color, and stress management in comparison with other

nutrients/micronutrients. Micronutrient deficiencies do not always show clear symptoms such as chlorosis, and determining micronutrient toxicities or deficiencies can be difficult. Actual deficiencies of Cu, Zn, Mn, B, Cl, Mo, and Ni are rarely reported in turfgrasses, but toxicities of Cu, Zn, Mn, Mo, and Ni can occur in areas contaminated by mining or other industry operations, soil amendments high in heavy metals, or excessive use of micronutrient fertilizers.

Sodium and Si can have effects on plant growth and development. Excess Na competes with other basic cations (K, Ca, and Mg mainly) for exchange sites and can cause leaching and ultimately deficiencies of these ions, in addition to its salinity stress to plants. Silicon is one of the most actively researched nonessential plant nutrient in recent years and some turf managers have adapted it as a turfgrass nutrient to be applied regularly to turf as a fertilizer.

New research is needed on turfgrass stresses associated with nutrients, particularly with micronutrients. ISM combined with the importance of environmental stewardship will continue to drive the need for further research in turfgrass nutrition and physiology, in addition to stress controls.

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# 39 Nutrient Management of Golf Course Putting Greens under Stresses

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## 39.1 INTRODUCTION

Golf has become a popular game since World War II in Western societies, and the number of golf courses in the world will continue to grow even though the world is currently facing economic challenges on a global level. Such a trend may be possible partially due to the facts that new golf courses have been rapidly developed within the last three decades in non-golf-traditional countries such as

Asian countries and other countries in the world. As game and sport, golf fits the broadest age spans from childhood to senior ages compared with other outside sports, and it has been a life-long hobby and exercise for many golfers. There are about 30,000 golf courses in the world and the economy is estimated at about \$50 to 60 billion each year just for the turfgrass management area. The golf course industry focusing on turfgrass management aspects includes more 1,000,000 part-time and full-time employees to maintain above mentioned 30,000 golf courses in the world by directly routine practices, providing supplies of grasses, fertilizers, chemicals, irrigation systems, golf course design and construction, and others (Beard, 2002; Haydu et al., 2008; McCarty, 2011).

Although the total putting green area for an 18-hole golf course is about 1 ha (10,000 m<sup>2</sup>) or about 1.6% of the whole golf course area (Beard, 2002), these putting greens absorb the most energy, cost, and labor for maintenance than other areas of a golf course (Dernoeden, 2000; Beard, 2002; McCarty and Miller, 2002; McCarty, 2011). Golf courses, sports fields, and lawns are specially monocultured functional crops. Among these functional turfgrass crops, golf course putting green management represents the highest level of turfgrass management. In addition, well-maintained putting greens represent the quality of the golf course with positive impacts to golfers, the game, community, environment, and local economy. Golf putting green turfgrasses are normally mowed at a range of 2.5–5.0 mm to meet the ball rolling requirement for playability and only a few turfgrass species from over 7500 species of the grass family, one of the four largest higher plant families, have been adapted as putting green turfgrasses (Table 39.1). Of course, the great potential is still there to explore new grass species (even from other plant families) for golf course and turf use and even suitable for future putting greens.

Due to the lower mowing height requirement, there are much fewer turfgrasses suitable for putting greens than other types of turf. The turfgrass species that have been adapted as putting green turfgrasses include both cool-season and warm-season turfgrasses (Dernoeden, 2000; Beard, 2002; McCarty and Miller, 2002; McCarty, 2011). Creeping bentgrass (*Agrostis stolonifera* L.), annual bluegrass (*Poa annua* L.), and velvet bentgrass (*Agrostis canina* L.) are the cool-season turfgrasses; rough bluegrass (*Poa trivialis* L.) is the most commonly used winter overseeding turfgrass; and the hybrids in *Agrostis* genus are future potential putting green turfgrasses. The dominant warm-season putting green turfgrass has been hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burt-Davy]. Seashore paspalum (*Paspalum vaginatum* Swartz.) and “Diamond” zoysiagrass [*Zoysia matrella* (L.) Merr.] (Engelke et al., 2002; Stiglbauer et al., 2009) are the relatively newer members of warm-season putting green turfgrasses with “Diamond” zoysiagrass as the newest, and have adapted in recent years. In comparison with hybrid bermudagrasses, these two new warm-season putting green turfgrasses have advantages of improved salinity, poor water quality, and shade tolerance used in the warmer climatic zones in the world (Table 39.1).

Although there are only a few of the turfgrass species available as putting green turfgrasses, there are much more varieties or cultivars under these species developed for putting greens. Creeping bentgrass is the species with the largest number of cultivars among these species because of its long history of use as a putting green turfgrass. To date, there have been several dozens of creeping bentgrass cultivars available, which still have been the dominant putting green turfgrass for cool climatic zones and the transition zones. With the efforts of bentgrass breeding and genetic research, the development of new hybrid bentgrass cultivars is promising to overcome current creeping bentgrass’ shortcomings of poor summer heat tolerance and high disease potential (Belanger et al., 2003, 2004; Bonos et al., 2006; Tian et al., 2009).

It has been exciting that additional four grass species, annual bluegrass, seashore paspalum, velvet bentgrass, and “Diamond” zoysiagrass, have become new members of putting green turfgrasses or more popular than they used to be. In the past several decades, the turfgrass breeders have successfully brought close to 1000 new turf cultivars and genetic lines to meet the turf industry’s demand from over a dozen turfgrass species ([www.ntep.org](http://www.ntep.org)). Therefore, the future new putting green turfgrass cultivars or species are promising.

**TABLE 39.1**  
**Major Stresses Encountered by Putting Green Turfgrasses and Improvement Potentials**

| Putting Green Turfgrasses             | Advantages                                                                                                                           | Main Challenges                                                                                                     | The Most Needed Improvement                                 | References                                                                                                                                                                               |
|---------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Cool-season</b>                    |                                                                                                                                      |                                                                                                                     |                                                             |                                                                                                                                                                                          |
| Creeping bentgrass                    | With the largest number of cultivars as a putting green species available, it serves the largest number of golf courses in the world | Summer heat, diseases, weed, thatch, and management in the transition zone from cool climate to subtropical climate | Summer heat resistance and disease resistance               | Xu and Huang (2000), Chai et al. (2002), Bonos et al (2003), Wang and Luthe (2003), Zhang and Ervin (2004), Fu et al. (2005, 2006), McCarty et al. (2005, 2007), and Bonos et al. (2006) |
| Annual bluegrass                      | Fresh green color and low mowing height tolerance                                                                                    | Summer heat and diseases                                                                                            | Diseases resistance                                         | Hagley et al. (2002), Huff (2003), Vargas and Turgeon (2004), Bertrand et al. (2009), Dai et al. (2009), Inguagiato et al. (2009), and Dionne et al. (2010)                              |
| Velvet bentgrass                      | Finest texture and green color                                                                                                       | Summer heat and some diseases                                                                                       | Disease resistance                                          | Brilman and Meyer (2000), Brilman (2003), and Koeritz and Stier (2009)                                                                                                                   |
| <i>Agrostis</i> hybrids               | Improved stress resistance to environment and diseases                                                                               | Unknown                                                                                                             | Further research and identification are needed              | Belanger et al. (2003), Belanger et al. (2004)                                                                                                                                           |
| <b>Warm-season</b>                    |                                                                                                                                      |                                                                                                                     |                                                             |                                                                                                                                                                                          |
| Hybrid bermudagrass                   | Excellent summer month performance in the transition, subtropical, and tropical zones                                                | Shade, thatch layer accumulation, winter dormancy, spring dead sport disease, salinity tolerance                    | Shade tolerance, disease resistance, and salinity tolerance | Carrow et al. (1987), McCarty and Miller (2002), McCarty (2005), Baldwin et al. (2006, 2009c), Zhang et al. (2006a,b), and Bauer et al. (2009)                                           |
| Seashore paspalum                     | Poor water quality and salinity resistance                                                                                           | Cold stress and lower mowing height                                                                                 | Cold resistance                                             | Duncan and Carrow (2000, 2005), Lee et al. (2005), and Kopeck et al. (2007)                                                                                                              |
| “Diamond” zoysiagrass                 | Shade tolerance and salinity resistance                                                                                              | Lower mowing height, thatch control, and diseases                                                                   | Zoysiagrass patch resistance                                | Qian and Engelke (1999), Engelke et al. (2002), Baldwin et al. (2009c), Chen et al. (2009), and Stiglbauer et al. (2009)                                                                 |
| <b>Winter overseeding turfgrasses</b> |                                                                                                                                      |                                                                                                                     |                                                             |                                                                                                                                                                                          |
| Rough bluegrass                       | Heat sensitive and fine texture                                                                                                      | Diseases and salinity stress                                                                                        | Diseases and poor water quality tolerance                   | Liu et al. (2001), Camberato and Martin (2004), Rajasekar et al. (2006), and Volterrani et al. (2009)                                                                                    |
| Perennial ryegrass                    | Fast germination                                                                                                                     | Diseases and salinity                                                                                               | Poor spring transition                                      | Volterrani et al. (2009)                                                                                                                                                                 |
| Annual ryegrass                       | Fast germination                                                                                                                     | Disease and salinity                                                                                                | Course texture                                              | Volterrani et al. (2009)                                                                                                                                                                 |
| Fine fescues                          | Heat sensitive                                                                                                                       | Unknown                                                                                                             | Unknown                                                     | Volterrani et al. (2009)                                                                                                                                                                 |

To maintain these putting green turfgrasses, fertilizers are needed (Turner and Hummel, 1992; Carrow et al., 2001; Throssell et al., 2009). It is much more challenging to manage these turfgrasses putting greens under a stress or multiple stresses than any other types of turf mainly due to the lowest mowing. Also, it is often the case that putting greens of a golf course are managed under a single stress, multiple stresses, a temporary stress, and/or permanent stress to overcome unfavorable growth conditions as long as the golf course is in use despite locations and climates. With new demands and more and more resource limitations, some of these stresses have become more critical than they used to be, such as water shortage and pesticide and fertilizer restrictions. Golf course management is a multiple-dimension approach, deals with all aspects of plant growth and culture, and overcomes all kinds of plant culture stress. This chapter attempts to (1) focus on and update putting green turfgrasses at the species level with an overall review of major stresses that putting green management has to encounter; and (2) highlight a possible approach of integrated stress management (ISM) related to nutrient management to successfully overcome putting green stresses (Liu et al., 2010, Chapter 38).

## 39.2 GOLF COURSE PUTTING GREENS

Sizes of individual putting greens range from less than 100 m<sup>2</sup> to several hundred square meters with the common sizes between 400 and 600 m<sup>2</sup>. Putting greens are also the most attractive areas of a golf course from the appearance point of view because of the lowest mowing height and smoothest surface maintained by frequent mowing than any other types of turf on the golf course. However, putting greens are often to encounter stresses. Due to the lowest mowing heights of putting greens, commonly seen stresses such as heat, cold, and drought are more severe for putting greens than other areas of golf courses even with the same turfgrass species and cultivars used.

In order to have a rapid drainage, the putting green root zone is often modified with sand to avoid waterlogging and assure enough pore spaces providing oxygen for root growth. There are two basic types of sandy root zone mix modifications for putting greens: pure sand greens, and sand and peatmoss mix greens. The pure sand root zone was first tested in California and it is also called California green. The California green has high water percolation rate and is suitable for rapid water drainage designed for areas with more heavy storms within a short period of times, but it has poor water and nutrient retention capability. The other type of sandy green is called USGA specification green because it was first invented by the United States Golf Association and revised a few times by using sand and peatmoss in different ratios by volume (United States Golf Association Green Section Staff, 1993). Normally, sand occupies 80%–90% by volume in the sand/peatmoss mix. USGA green overcomes the shortage of rapid water percolation and poor nutrient retention and it is suitable for the majority of areas in the world. Native soil and partially modified putting greens are also found with a challenge of poor subsurface water drainage as an economic way to build new putting greens (Beard, 2002; McCarty, 2011).

The putting green management includes the primary practices of mowing, fertilization, and watering, and in addition pest control, cultivation, and chemical applications of plant growth regulators, surfactants, and biostimulants. Other practices are often needed to maintain putting greens, such as dew and surface water removal; winter overseeding or painting; surfacing rolling; winter or summer covering; microclimatic and growth condition modification by the use of fans to decrease air temperature and increase air circulation; SubAir systems to remove excessive water and improve temperature extremes of root zones; and hydronics ([www.subairsystems.com](http://www.subairsystems.com)), a subsurface tubing system to increase or decrease root zone temperatures by running through hot and cool water during winter or summer seasons to extend putting green turfgrass growth and color (Beard, 2002; McCarty, 2011).

## 39.3 GOLF COURSE PUTTING GREEN TURFGRASSES

Table 39.1 lists the current putting green turfgrasses, and they belong to cool-season or warm-season turfgrasses. Creeping bentgrass is the most dominant cool-season turfgrass used on putting greens because of its fine texture, stoloniferous growth habit, and low mowing-height tolerance (Warnke, 2003).

Annual bluegrass serves areas without severe summer heat in the cooler climate zones than creeping bentgrass (Huff, 2003; Vargas and Turgeon, 2004). Velvet bentgrass (Brilman and Meyer, 2000; Brilman, 2003) has the finest leaf texture among all these putting green turfgrasses and is the least popular cool-season putting green turfgrass so far. It has similar characteristics as annual bluegrass to adapt to cool climate without severe summer heat, and it may have the potential of less disease potentials than creeping bentgrass (Koeritz and Stier, 2009). Rough bluegrass is often used as winter overseeding for warm-season putting green turfgrasses in the warm climate zones (Hurley, 2003). These cool-season turfgrasses are suitable for cool and cold climatic zones including the transition zone with a challenge of summer heat stress but with an advantage of winter month play without overseeding requirement as warm-season turfgrasses do.

Warm-season putting green turfgrasses include hybrid bermudagrasses, seashore paspalum, and “Diamond” zoysiagrass (Duncan and Carrow, 2000; Beard, 2002; Engelke et al., 2002; Kopec et al., 2007; Stiglbauer et al., 2009; McCarty, 2011). These warm-season turfgrasses are found on golf courses in hot tropical, subtropical, and warm climatic zones. Warm-season turfgrasses can be used in the transition zone with the challenge of winter kills but awarded with the advantage of no heat stress during the hot summer season. Hybrid bermudagrasses and seashore paspalum have several cultivars used for putting greens. To date, “Diamond” zoysiagrass has been the only zoysiagrass used for putting greens in the United States as the newest member of putting green turfgrasses. However, some other putting green zoysiagrasses have been found in its origin countries in Asia.

## **39.4 SELECTED STRESSES ASSOCIATED WITH PUTTING GREEN TURFGRASSES**

The most common stress of cool-season putting green turfgrasses is the summer heat and high disease potential, while among the warm-season putting green turfgrasses, main stresses vary (Dernoeden, 2002; McCarty and Miller, 2002; McCarty, 2011). Shade and spring dead spot disease are the most limiting factors for hybrid bermudagrass putting greens in many locations. The most serious stresses for seashore paspalum and “Diamond” zoysiagrass as putting green turfgrasses have not been identified because of relatively fewer number of golf course use and shorter history as putting green turfgrasses. However, seashore paspalum is relatively less cold hardy than zoysiagrass and bermudagrass, and that limits its distribution. Zoysiagrass often has a high brown patch disease potential, and brown-patch-resistant zoysiagrass has not been made available yet. All these putting green turfgrasses have to overcome one or multiple stresses during growing seasons. For example, these turfgrasses have adapted to low mowing heights, and low mowing heights are directly or indirectly involved with severity of many stresses. In other words, mowing heights of 2.5–3.2 mm are never favorable for any types of grasses but serve well for golf game, and there is no stress-free putting green turfgrass.

### **39.4.1 SUMMER HEAT STRESS**

High temperature limits the growth of cool-season turfgrasses during summer in many areas in the world because cool-season turfgrasses are  $C_3$  plants and grow most actively within a temperature range of 15°C–25°C (Beard, 1973; DiPaola, 1984; DiPaola and Beard, 1992, Fry and Huang, 2004). All nutrients are important for cool-season turfgrasses; during the summer, heat stress and high-temperature stress inhibit photosynthesis, limit carbohydrate accumulation, damages cell membranes, cause protein folding, and even lead to cell death (Fry and Huang, 2004). Reduction of fertilizer input, particularly with N, is highly recommended for cool-season putting green turfgrasses during summer months to reduce the potential for fertilizer burn and root injury. Light and frequent foliar fertilizer input assure the summer turf quality, and all cool-season turfgrasses have the ability to acquire heat tolerance in certain degrees by exposure to a gradual increase in temperature, which

happens naturally as heat acclimation (Fry and Huang, 2004; Totten et al., 2008). Under heat stress, cool-season turfgrass root systems are more negatively affected than leaves (Liu et al., 2002) and liquid foliar nutrient application may be the only choice to provide cool-season turfgrasses nutrients to avoid or minimize any fertilizer salt burning and injury to roots (Liu et al., 2008a,b).

Extremes to avoid any N and other nutrient input during the stressful summer heat tend to further increase heat stress leading to further carbon depletion (Fry and Huang, 2004; Shen et al., 2009; Zhang et al., 2010). Foliar and light nutrient applications are highly recommended for highly maintained turf including golf course putting greens. The reasons for nutrient requirement under heat stress may include (1) to maintain basic metabolisms to survive, and (2) to activate mechanisms for heat-tolerant cultivars to overcome heat stresses including formation of heat-shock proteins (HSPs), hormone regulations, and antioxidant enzymes production (Taiz and Zeiger, 2006). These formations of and adjustments are all directly or indirectly associated with proper nutrient supply. However, proper nutrient supply in enhancing these heat resistance mechanisms need to be further investigated for turfgrasses, particularly for  $C_3$  cool-season turfgrasses (Liu et al., 2008a,b).

Wang and Luthe (2003) found that heat-sensitive creeping bentgrass variants fail to accumulate chloroplast HSP isoforms. The role of chloroplast-localized small heat-shock proteins (CP-sHSPs) in heat tolerance is to provide protection to photosystem II during heat stress. The accumulation of the additional CP-sHSP isoforms is genetically linked to heat tolerance and the presence of the additional isoforms in the heat-tolerant creeping bentgrass variants indicates that the heat-tolerant creeping bentgrass can be enhanced at genetic and biochemical levels. Additional nutrient supply may not be significant to enhance HSP formation and expression but nutrient deficiency may negatively affect the heat tolerance performance of heat-resistant turfgrasses during hot summer months in addition to carbohydrate depletion losses.

With the situation of most sandy soil culture of putting green turfgrasses, Ca rarely becomes deficient. However, Ca, as a signal plant nutrient, has to be studied for its role to reduce heat stress particularly for  $C_3$  plants during hot summer months intensively in recent years. How practically to use Ca to enhance  $C_3$  turfgrass heat tolerance is unclear and requires further investigations (Jiang and Huang, 2001; Fu and Huang, 2003; Saidi et al., 2009).

Although there is lack of documented research, high soil moisture content and organic matter in root zones during hot summer months may negatively affect  $C_4$  warm-season turfgrass nutrient uptake, even warm-season turfgrasses are not under heat stress (Brecht et al., 2009). However, with lower mowing heights and high potential of thatch accumulation, smaller root systems are often found from warm-season turfgrass putting greens during the summer months and foliar nutrient applications also benefit warm-season turfgrasses nutrient uptake during hot summer months. For putting green turfgrasses, heat, drought, and salinity stresses are often associated with each other due to climatic conditions and poor soil and water supplies.

For both cool- and warm-season putting green turfgrasses, nutrient management of N, K, Ca, Si, Na, and Fe (Liu et al., 2008a,b, [Tables 39.2](#) and [39.3](#)) can be significant in reducing heat stress, which is often combined with drought and salinity stresses during hot summer months. Approaches with plant growth regulators (Xu and Huang, 2010) and biostimulant input for creeping bentgrass summer heat stresses can enhance heat resistance and indirectly favor nutrient use and metabolisms under heat stress (Ookawa et al., 2004; Xu and Huang, 2009; Zhang et al., 2010).

### 39.4.2 SHADE STRESS

In general,  $C_3$  plants do not need whole day direct full sunshine and  $C_4$  plants almost need all sunlight they can get with a tolerance to high solar radiation during growth seasons. However, today's putting green turfgrasses respond to shade stress differently and they cannot be simply separated just based on whether they are  $C_3$  cool-season or  $C_4$  warm-season turfgrasses. The newest member of putting green turfgrass, "Diamond" zoysiagrass, can tolerate low light intensity as much as 70% shade without losing its putting green quality. With plant growth regulator trinexapac-ethyl (TE)

**TABLE 39.2**  
**Major Turf Stresses Associated with Fe Status and Availability**

| Stress/Fe Status,<br>Recommendations,<br>and Future<br>Improvements                            | Management<br>Recommendations<br>to Reduce Stresses<br>and Enhance Fe<br>Turf Use |                          | Evidences or<br>Potentials for<br>Improvement                                                                                      | References                                                                                                                                                        |
|------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|--------------------------|------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                                | Fe <sup>a</sup> Deficiency                                                        | Fe <sup>a</sup> Toxicity |                                                                                                                                    |                                                                                                                                                                   |
| Sandy alkaline soils<br>with soil pH > 7.0                                                     | >/-<br></+                                                                        | >/+<br></-               | Light and frequent<br>foliar Fe<br>applications                                                                                    | Centipedgrass,<br>creeping<br>bentgrass,<br>annual<br>bluegrass<br>Carrow et al. (1988,<br>2001), Turner and<br>Hummel (1992),<br>and Xu and<br>Mancino (2001)    |
| High soil P contents<br>and waterlogging<br>conditions                                         | >/-<br></+                                                                        | >/-<br></+               | Reducing P<br>fertilizer input and<br>more frequent Fe<br>applications, and<br>reduce Fe input<br>during<br>germination            | Arabidopsis, rice<br>Ward et al. (2008),<br>Fageria et al.<br>(2008), and Zheng<br>et al. (2009)                                                                  |
| Drought/heat stress                                                                            | >/-<br></+                                                                        | >/+<br></+               | Iron forms plus<br>plant growth<br>regulators                                                                                      | Creeping<br>bentgrass<br>Glinski et al. (1992),<br>Zhang et al. (2002)                                                                                            |
| Cold soil<br>temperatures and<br>fall and winter turf<br>color                                 | >/-<br></+                                                                        | >/?<br></?               | Light foliar<br>applications                                                                                                       | Bermudagrasses,<br>creeping<br>bentgrass,<br>Kentucky<br>bluegrass<br>White and Schmidt<br>(1989, 1990), Zhang<br>et al. (2002), and<br>Devetter et al.<br>(2008) |
| Imbalanced soil<br>nutrients including<br>higher heavy metal<br>contents Zn, Cu,<br>Mn, and Cd | >/-<br></+                                                                        | >/+<br></?               | Reducing heavy<br>metal nutrient<br>element input,<br>reduce heavy<br>metal input<br>sources, and use<br>Fe efficient<br>cultivars | Wheat, rice<br>Takahashi (2003),<br>Chen et al. (2004),<br>and Ghasemi-Fasaei<br>and Ronaghi (2008)                                                               |
| Black layer                                                                                    | >/?<br></+                                                                        | >/-<br></+               | Reducing Fe and S<br>input and<br>improving poor<br>soil drainage<br>conditions                                                    | Cool-season<br>turfgrass<br>putting greens<br>Hodges (1992),<br>Vargas (2005), and<br>Berndt and Vargas<br>(2006, 2008)                                           |

<sup>a</sup> For each pair of signs separated by “/,” the less, greater, or “?” signs on the left indicate the stress severity decreased, increased, or no effects, respectively; the positive or negative signs on the right side indicate with or without Fe application or toxicity level respectively. The “?” sign indicates currently unknown to the authors’ knowledge.

applied regularly under shade, “Diamond” zoysiagrass shade tolerance can be improved even under 90% shade (Qian and Engelke, 1999; Baldwin et al., 2009d).

Except for hybrid bermudagrasses, in general, cool-season putting green turfgrasses plus sea-shore paspalum and “Diamond” zoysiagrass can tolerate partial shade. Reducing nutrients has been recommended for both warm- and cool-season turfgrasses under shade stress because of a low ratio of photosynthesis: respiration that reduces turf growth and divot recovery (Beard, 1997; Carrow et al., 2001). Selecting shade-tolerant species and adjusting management techniques are the



**TABLE 39.3**  
**Major Putting Green Turfgrass Stresses and Possible and Potential Association with Si Applications for the Future**

| Putting Green Turfgrass Stresses | Stress Severity <sup>a</sup> /Si Application | Forms of Si Applied to Turfgrasses or Other Crops                                                                  | Evidences or Potentials for Improvement                                   | References                                                                                                                           |
|----------------------------------|----------------------------------------------|--------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Salinity                         | >/-<br></+                                   | CaSiO <sub>3</sub> , Na <sub>2</sub> Si <sub>3</sub> O <sub>7</sub>                                                | Sugarcane, grape                                                          | Ashraf et al. (2010), Soylemezoglu et al. (2009)                                                                                     |
| Heavy metal or element toxicity  | >/-<br></+                                   | K <sub>2</sub> SiO <sub>3</sub> , H <sub>4</sub> SiO <sub>4</sub> , Na <sub>2</sub> Si <sub>3</sub> O <sub>7</sub> | Al, As, Mn, Cd, B                                                         | Liang et al. (2005), Guo et al. (2007), Doncheva et al. (2009), Exley (2009), Soylemezoglu et al. (2009), and Vaculíka et al. (2009) |
| Drought                          | >/-<br></+                                   | K <sub>2</sub> SiO <sub>3</sub> , CaSiO <sub>3</sub>                                                               | Grasses, sorghum, cucumber, St. Augustinegrass                            | Trenholm et al. (2004), Eneji et al. (2008), Hattori et al. (2008), and Sonobe et al. (2009)                                         |
| Cold                             | >/-<br></+                                   | K <sub>2</sub> SiO <sub>3</sub>                                                                                    | Wheat                                                                     | Liang et al. (2008)                                                                                                                  |
| Heat                             | >/-<br></+                                   | CaSiO <sub>3</sub>                                                                                                 | Bermudagrass                                                              | Brecht et al. (2009)                                                                                                                 |
| Diseases                         | >/-<br></+                                   | H <sub>4</sub> SiO <sub>4</sub> , CaSiO <sub>3</sub>                                                               | <i>Bipolaris cynodontis</i> , brown patch, grey leaf spot, powdery mildew | Saigusa et al. (2000), Brecht et al. (2007), Nanayakkara et al. (2008), and Brecht et al. (2009)                                     |
| Insects                          | O/-<br></+?                                  | K <sub>2</sub> SiO <sub>2</sub>                                                                                    | <i>Rusidrina depravata</i> Butler, aphids                                 | Saigusa et al. (2000), and Ranger et al. (2009)                                                                                      |
| Turf wear stress                 | >/-<br></+                                   | K <sub>2</sub> SiO <sub>2</sub>                                                                                    | Seashore paspalum                                                         | Trenholm et al. (2001)                                                                                                               |
| Thatch layer accumulation        | ?/-<br>?/+                                   | ?                                                                                                                  | ?                                                                         | ?                                                                                                                                    |

<sup>a</sup> For each pair of signs separated by “/,” the less, greater, or “O” signs on the left indicate the stress severity decreased, increased, or no effects, respectively; the positive or negative signs on the right side indicate with or without Si application respectively. The “?” sign indicates currently unknown to the authors’ knowledge.

two approaches commonly recommended for maintaining turf in reduced light conditions. Raising mowing height to alleviate shade stress is commonly recommended as well.

Among all the nutrients, N reduction for shade management is critical. Goss et al. (2002) compared liquid applications of N to confirm that lower N rates (150–185 kg ha<sup>-1</sup> annually) resulted in better quality turf than higher N rates (212–235 kg ha<sup>-1</sup>). Granular forms of N must be dissolved in soils first then are absorbed through roots and transported to shoots of a plant, a process that could be energy inefficient by forcing roots to use their carbohydrates for energy to assimilate and transport N to the shoots (Hull and Liu, 2005). In a shaded environment, turfgrass root development and energy budgets are stressed due to low photosynthetically active radiation. Since the majority of N is utilized in the shoots, increased foliar absorption from liquid applications of N may increase N-use efficiency and allow more photosynthate to be allocated to the roots of the plant, enabling the turfgrass to attain more nutrients and water (Beard, 1997). Although research reports indicate that 100% liquid N and a combination of liquid N and granular N helped to improve turf quality (Totten et al., 2008), comparisons between granular and liquid N applications on shaded turf have been rarely reported.

In addition to N, Fe has been investigated. Baldwin et al. (2009c) reported that “Champion” bermudagrass putting green quality can be enhanced under 55% light reduction maintained at a 3.2 mm mowing height under reduced sunlight. Fe applications had minimum effects to reduce shade stress but TE improved shade tolerance. Reduced N input benefited shade condition surviving with a reduced thatch accumulation. Baldwin et al. (2009a) also identified that winter month moderate shade (<60%) to a creeping bentgrass green was not detrimental and Fe effects were not significant to enhance shade tolerance during winter months, and a similar result was found for rough bluegrass overseeded on “Champion” bermudagrass putting green (Baldwin et al., 2009c).

Another technique to improve turf in shaded conditions is the application of plant growth retardants (PGRs), particularly TE, which inhibits gibberellic acid biosynthesis. In addition to reducing clipping yields, multiple applications of PGRs increased turf density, color, and quality of both cool-season and warm-season turfgrasses. In addition, increased tillering and density of turf mowed at golf green height under shade were found for both warm- and cool-season turfgrasses, but turf quality was still unsatisfactory due to low irradiance for most warm-season turfgrasses, particularly hybrid bermudagrasses, when the light reduction is beyond 60% (Qian and Engelke, 1999; Baldwin et al., 2009c).

Shade is still the number one limiting factor for hybrid bermudagrass putting greens close to trees under warm climatic conditions. Severe shade is even detrimental to hybrid bermudagrasses (Bunnell et al., 2005a–c; Baldwin et al., 2009a). However, the recently released new shade-tolerant hybrid bermudagrass cultivar encourages further shade tolerance improvement for bermudagrasses (Hanna et al., 2010).

There is still a lack of research on seashore paspalum shade tolerance as a putting green turfgrass, but several other studies have demonstrated that seashore paspalum has a moderate shade tolerance in comparison with hybrid bermudagrasses (Jiang et al., 2004; Baldwin et al., 2009d). Golf course putting green shade is most likely caused by trees and shade intensity; penetrated light quality from tree canopies and tree species and varieties affect the outcome of light quality received on putting greens. Afternoon shade is more detrimental than morning shade for hybrid bermudagrass putting greens, and there is no difference for creeping bentgrass putting green (Bell and Danneberger, 1999; Bunnell et al., 2005a–c; Baldwin et al., 2009a). A greenhouse study demonstrated that “Diamond” zoysiagrass and “Seadwarf” seashore paspalum had better growth and appearance than both hybrid and common bermudagrasses with treatment of blue, red, and yellow tarps for 8 weeks (Baldwin et al., 2009d).

### 39.4.3 DISEASE STRESS

Nutrient enhancement of putting green turfgrasses under disease stresses can be complicated, but it is promising (Hull et al., 1979; Huber and Arny, 1985; Reuveni and Reuveni, 1998; Datnoff, 2005; Dordas, 2008; Ghorbanim et al., 2008). Nutrient enhancement does not simply mean greater quantity of input; it means proper application with the understanding of the nature of stresses. There are much more research reports available on the effects of plant nutrients on diseases severity than the nutrient enhancement for diseased plants. It may be partially due to the fact that the majority of the studies were based on annual yield production crops and those diseased crops had much less recovery potential from the yield losses than perennial function crops such as turfgrasses to recover from diseases. Therefore, the concept of nutrient enhancement for stressed turfgrasses is much more important and practical as a part of ISM (Liu et al., 2010, Chapter 38).

Dollar spot is the most common putting green disease and fairy rings happen on all kind of putting greens. Cool-season putting green turfgrasses have several serious diseases including pythium blight, brown patch, snow molds, and summer patch in addition to dollar spot. So far, seashore paspalum has not been found with its unique disease, but hybrid bermudagrass and zoysiagrasses have been affected more often by spring dead spot and large brown patch, respectively.

Due to the lower mowing height, putting greens are the type of turfgrasses most vulnerable to diseases, and the expenses to prevent and control putting green diseases can be a significant budget for golf course management. Nitrogen has been most commonly studied as a nutrient element in relation to plant diseases. Excessive N applications encourage turfgrass succulent growth and delayed dormancy, which may increase the susceptibility to pathogens and other stresses such as winter kill or summer decline. On the other hand, nitrogen deficiency weakens turf vigor and increases susceptibility to pathogens. Nitrogen form, source, rate, and time of application play important roles in turfgrass diseases. The relationships between N application practice and disease severity and disease occurrence among turfgrasses are rather complicated by the existence of host vertical and horizontal resistances to diseases, epidemiology of different diseases, coexistence of multiple diseases, and different host recovery mechanisms. In general, two groups of turf diseases are affected by N application rate: disease severity increased and disease severity reduced plus a group of diseases not affected by N application rate or lack of information (Liu et al., 2008a,b).

Although limited information is available on mechanisms of reduced severity of diseases by N application, turfgrasses have been reported either with improved resistance or speedy recovery of foliar diseases including dollar spot (*Sclerotinia homoeocarpa* F. T. Bennett), leaf spot (*Drechslera* spp.), red thread [*Laetisaria fuciformis* (McAlpine) Burdsall], rusts (*Puccinia* spp.), and foliar anthracnose [*Colletotrichum graminicola* (Ces.) G. W. Wils.]. Recent studies indicate foliar N application can even more efficiently reduce or prevent these foliar diseases.

The worst group of turfgrass diseases are soil-borne crown diseases including pythium blight [*Pythium aphanidermatum* (Edson Fitzp.)], brown patch (*Rhizoctonia solani* Kühn), gray snow molds (*Typhula incarnata* Fr.), gray leaf spot [*Pyricularia grisea* (Cooke) Sacc.], dead spot (*Ophiosphaerella agrostis*), and spring dead spot (*Ophiosphaerella* spp.), which are easily induced or worsened by heavy N rates, particularly when inorganic quick-release N fertilizers are used. Excessive application of N promotes thinner cell walls, more succulent tissues, and less carbohydrate reserves, which encourage pathogen's penetration to the host plants.

The influence of the two major N forms of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  on crop diseases have been intensively studied, and the two N forms normally have opposite effects to particular diseases. The influences of N form on disease severity include impacts to the host, pathogen, and the soil environment. The effect of specific N form on disease severity depends on many factors. Susceptible cultivars or species are easily affected by N forms while resistant turfgrasses are not affected by diseases regardless of N forms.

Practices of N application to turf are rather more important than N nutrient itself for encouragement or discouragement of turf diseases. Improper application time, rate, and N form and sources will promote disease occurrence. For example, N fertilizers have effects on soil pH and some turfgrass diseases are soil pH sensitive. Alkaline soil conditions promote take-all patch [*Gaeumannomyces graminis* (Sacc.) Arx & D. Oliver var. *avenae*] and slight acidic soils suppress most of the patch diseases. There is a lack of evidence of N forms on the aggressiveness of turfgrass pathogens.

Limited information is available for the interaction between fertilizer application and non-fungal pathogenic turf diseases such as nematodes, viral diseases, and bacterial wilts for turfgrasses. However, recent findings suggest that the other symbiotic relationship between host and endophyte may provide another possible dollar spot control method. Alumai et al. (2006) reported that entomopathogenic nematodes correlated significantly with sand, silt, P, organic matter, and Mg content, but not with clay, pH, Ca, and K.

Although short of quantitative data, golf course putting greens have the highest disease potential whether using creeping bentgrass, bermudagrass, or zoysiagrass because of the lowest mowing height and intensive traffic in comparison with any other types of turf. Nitrogen plays important roles in physical damage recovery to turf. Typically, golf course tees and sports fields receive more damages than golf putting greens, but tees and sports fields can recover quicker than greens due to the higher mowing height and much less pressure for diseases. The better reserves in roots, crowns, and other parts of higher mowing turf benefit a quicker recovery from damages with a relatively less

N requirement comparing with a lower and more frequently mowed turf. Due to the controversial responses to N for turf diseases, the zone for optimum N application is very restricted for most turfgrasses (Liu et al., 2008a,b). In the middle zone, there is still a group of diseases independent to N status and supplies or short of information.

Other mineral nutrient management has been applied to reduce crop and turfgrass diseases or as a remedy to cure crop or turfgrass diseases, including copper, manganese, silicon, and potassium by either enhancing turf resistance to pathogens or suppressing pathogen infections. However, nutrients themselves may never be an efficient tool to cure turfgrass diseases directly but the complicated roles of nutrients in different diseases deserves much more attention for management and future turfgrass improvement. For example, a selected genetic line of creeping bentgrass with natural resistance to dollar spot may also have greater capability to absorb N efficiently by roots and leaves. Nitrogen effects to nonpathogenic microbial activities exist, and these effects may have an influence on the turf quality.

#### 39.4.4 WEED STRESS

Turfgrasses compete with weeds for nutrients, and different soil nutrient levels also favor one plant species over another. It is hard to use soil nutrient levels and fertilizers as remedies to control weeds in turf and it will be unlikely a main tool in weed control. The difference of nutrient uptake efficiency between a weed and turfgrass species or cultivar can be further enhanced. There are generally three aspects to enhance nutrient use efficiency in weed suppression or control for turf: (1) implementing sound nutrient programs to maximize turf vigor to reduce weed potential; (2) applying different levels of nutrients to favor turfgrass and suppress weed species with understanding the nutrient requirement differences between turfgrasses and weed species; and (3) generating toxic nutrient or chemical levels (non-herbicide chemicals) to control weeds without damaging the turf species.

Weeds can take advantage of a turf with a better resistance to stresses including nutrient deficiencies. The combination of proper nutrient balance associated with other cultural practices ensures long-term turf vigor, which may minimize weed potentials. It is very common for weed invasion to start in a turf under stress or at the early establishment. Documented research is limited on competitions between turfgrass species and cultivars and weed species; and among multiple turfgrass and weed species on nutrient forms, supplies, and sources. However, under N deficiency, legumes that can fix N such as clovers (*Trifolium* spp.), black medic (*Medicago lupulina* L.), common lespedeza [*Kummerowia striata* (Thunb.)], along with grassy weeds such as bromesedge (*Andropogon virginicus* L.), quackgrass (*Agropyron repens* L.), bahiagrass (*Paspalum notatum* L.), and centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.] can be more troubling weeds for the turf. These weeds do not need or require very little N input and can survive well under N deficiency.

Under nutrient deficiency, turfgrasses lose their vigor to compete with weeds and become worse under stresses of drought, temperature extremes, nutrient deficiency, other pests, and traffic impacts. With weed existence, the actual available nutrients to the turfgrass will be reduced depending on the degree of weed coverage. It is most likely the worse the weed coverage, the worse the nutrient stresses to the turf in addition to the differences between the turfgrass and weed nutrient uptake capabilities. Some broadleaf weeds may have further advantages with deeper tap root systems for nutrient acquisition than grasses having relatively shorter fibrous root systems. Although there is a lack of research, it is not difficult to predict that some weeds may have advantages to absorb nutrient from soils at very low concentrations (<0.5 mM), at where some turfgrass species may already stop to absorb nutrient. Furthermore, recent studies by Hossain et al. (2004) and Sistani et al. (2003) showed much aggressive N and phosphorus (P) uptake by grassy weeds of torpedograss (*Panicum repens* L.) and southern crabgrass [*Digitaria ciliaris* (Retz.) Koel.]. The capability to accumulate N in the green tissues also further indicates a high N uptake efficiency by crabgrass species.

The optimum to excessive N supplies encourage several weed species occurrence including annual bluegrass (*Poa annua* L.), bentgrasses (*Agrostis* spp.), bermudagrasses (*Cynodon* spp.), ryegrasses (*Lolium* spp.), and crabgrasses (*Digitaria* spp.) plus several annual broadleaf weeds. Encroachments between turfgrass species are also encouraged by favorable nutrient supply for the more aggressive species in addition to other conditions and their own characteristics. For example, the more commonly observed encroachment is the surrounding bermudagrass invasion to creeping bentgrass putting greens; however, the opposite invasion was observed partially due to the aggressive N fertilization program to the creeping bentgrass green versus the surrounding N-stressed bermudagrass. Excessive N supply to turf promotes shoot, stolon, and rhizome growth with relative smaller root systems, less carbohydrate reserves, more succulent tissues, and more vulnerability to some diseases and insects, which contribute to a weakened vigor for the turfgrass to compete with weeds.

Turfgrasses also can lose their competitiveness with weeds under excessive nutrient supplies. At high external nutrient ion concentrations (normally  $>1.0\text{ mM}$ ), the  $V_{\text{max}}$  plateau of ion uptake kinetics is exceeded as a low affinity transport system (LATS) becomes functional. Therefore, the uptake rates increase in a linear function with ambient nutrient concentrations (between 5 and  $100\text{ mM}$ ) and exhibit no evidence of saturation kinetics. Some weeds have superior LATS systems in absorbing nutrients than turfgrasses when excessive nutrients are applied. Mowing practice adds complexity for nutrient effects on turfgrass and weed competitions. Although both turfgrasses and weeds are normally encouraged by adequate nutrient input, proper mowing practices enhance turf quality and suppress weeds more significantly particularly for broadleaf weeds depending on turfgrass species and weed species.

Turf thatch accumulation is encouraged by excessive N applications. Thatch normally provides a physical barrier from weed invasion particularly from seed germination. Under proper nutrient supply, thatch functions positively to suppress weed invasion. A turf becomes more vulnerable to weed invasion after aerifications. Weeds germinate much easier on bared soils than on a turf with proper moisture and temperature. Some turfgrass species may naturally have allelopathic effects to weeds by producing toxins or having aggressive growing habits. However, there is a lack of evidence on nutrient roles for either allelopathic effects of turfgrasses and the relationship between nutrient supply and enhancement of allelopathy.

Nutrient sources and forms also have ecological impacts on turf and weed competition. In general, annual summer grassy weeds will be encouraged with inorganic and quick-release fertilizers. Perennial grassy weeds with the closest similarity to turfgrasses are more difficult to control by cultural practices. The use of ammonium-type N fertilizers and acidic fertilizers with a potential to reduce several patch diseases for turfgrasses has been encouraged. Excessive application of ammonium-type and acidic fertilizers may lead to a lower soil pH with reduced available P, Ca, and Mg, and under such a soil condition, annual bluegrass as a weed is discouraged since it needs more P and a neutral soil pH than the desired creeping bentgrass.

Nutrients cannot serve as remedy to control weeds effectively for turf, but a proper fertilization program plays an extremely important role in association with other cultural practices to keep a strong turf vigor for healthy conditions to minimize weed invasion. Corn gluten meal has been used for both grassy and broadleaf weed control by suppressing weed seedling germination. Corn gluten meal on average contains 60% protein and 10% N by weight and also functions as N fertilizers. The amino acids as dipeptide forms in corn gluten meal mainly inhibit new weed seedling root germination and growth with negligible phototoxic effects on the existing turf. Brosnan et al. (2009) reported that a single granular application of fine salt (99% sodium chloride, 1% sodium silicoaluminate) at a rate of  $1464\text{ kg ha}^{-1}$  provided 84% and 23% control of sourgrass (*Paspalum conjugatum*) 6 weeks after initial treatment in seashore paspalum turf in Hawaii. Using sodium chloride (NaCl) as a weed control may only apply to salinity-tolerant turfgrasses as seashore paspalum, which can tolerate salinity levels as high as  $54\text{ dS m}^{-1}$ , a level at which most turfgrass species cannot survive (Duncan and Carrow, 2000). Wiecko (2003) reported that applications of saline ocean water

(EC = 55 dS m<sup>-1</sup>) controlled large crabgrass (*Digitaria sanguinalis* L.) in seashore paspalum, but repeated applications of saline ocean water did not control yellow nutsedge (*Cyperus esculentus* L.).

Dandelions (*Taraxacum officinale* Weber), a very popular broadleaf weed on turf worldwide showed different responses to N and K. Johnson and Bowyer (1982) reported that after 4 years of fertilization treatments, dandelion cover was reduced when fertilized with a higher rate N of 600 kg ha<sup>-1</sup> year<sup>-1</sup> than at a lower rate N of 300 kg ha<sup>-1</sup> year<sup>-1</sup> in Kentucky turf, while Tilman reported that dandelion was a poorer competitor for potassium than cool-season turfgrasses such as *Festuca* species.

Fertilization time and methods affect weed and crop competition. Dunn et al. (1993) reported in the northern transition zone that N fertilizer applications later than September encouraged winter annual weed emergence, during the time that zoysiagrass would normally become dormant. In addition, any fertilization during the slow growing season for the turfgrass may be more favorable for weeds (Busey, 2003).

Excessive nutrient supply can be toxic to plants by interrupting regular physiological metabolisms. The sensibility to nutrient toxicity between turfgrasses and weeds deserves more attention. There is a lack of literature of synergetic effect between plant nutrients and herbicides for turfgrass weed control; even many herbicides contain nutrient elements. However, an antagonism may happen when nutrients and herbicides are co-applied. Scroggs et al. (2009) reported that in the field studies, weed control was greatest when glyphosate was applied alone with control of barnyardgrass, browntop millet, and Palmer amaranth ranged between 93% and 95%. When glyphosate was co-applied with formulations of zinc sulfate, control of these three weed species was reduced to 39%, 39%, and 45%, respectively. These results indicate that glyphosate-based weed control is reduced when co-applied with the zinc products at their current use rates. Antagonism of the herbicidal performance of glyphosate from other cations such as Fe, Ca, Mg, Na, and K were also observed.

The future nutrient related research enlightens areas such as turf-weed ecology with a better understanding of nutrient uptake efficiency and nutrition physiology between turfgrasses and weeds; impacts of nutrient application rates, fertilizer forms, and sources, and time and method of application to the turf and weed competition; and helpful nutrient management programs for turfgrasses under weed stresses.

Quick-release nitrogen fertilizers have been applied to overseeded warm-season putting greens to speed spring transition, and the fertilizers will speed up the spring green up of the warm-season turfgrass and suppress the overseeded cool-season turfgrass with high N fertility to spring high temperatures.

#### 39.4.5 SALINITY STRESS

Salinity is an important growth-limiting factor for most turfgrasses, which are non-halophytic plants. Excessive salts in soils inhibit turfgrass growth by osmotic stress, nutritional imbalance, and specific ion toxicity in addition to the structural damages to soils. Soil salinity can be progressively exacerbated by practices such as irrigation and fertilization, especially in arid regions. The proper use of N fertilization in saline soils is important to sustain N supply for turfgrasses. On the other hand, over fertilization with N may contribute to soil salinization and increase the negative effects of soil salinity on turf performance.

The two major salinity sources for turfgrasses are natural salinity conditions of soils and irrigation water with high salt contents applied to turfgrasses. The relationship between turfgrass salinity stress and N fertilization deserves much more attention because of the total areas of turf under salinity stress and the complicated soil chemical interactions among Na<sup>+</sup>, Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, other N forms, cations, and anions in soils. Turfgrasses demonstrated diversified salinity tolerance, and salinity-tolerant plants seem less affected in N uptake and metabolism than salinity-sensitive plants.

Putting green turfgrass responses to salinity may change with mowing height, turf use, seasons, and nutrient input. To date, there is a lack of proper recommendation of specific fertilizer for each putting green turfgrasses to either salinity-sensitive or tolerant turfgrass species.

Studies of turfgrass growth responses to N and soil salinity for different turfgrass species and turf use are important to reveal whether the amount of N applied alleviates or aggravates the detrimental effects of salinity. In addition, examining turfgrass growth during different season associated with other stresses may provide profound information to practitioners to enhance salt tolerance over time such as winter overseeding and association with other pest stresses. Under salinity stresses, both  $\text{NO}_3^-$  and  $\text{NH}_4^+$  uptake were affected in tall fescues. Ammonium compared to  $\text{NO}_3^-$  nutrition is known to increase the salinity sensitivity in maize, wheat, and peas. Under both N and salinity stress, shoot growth is much reduced than roots.

#### 39.4.6 LOW-TEMPERATURE STRESS

It has been well documented that increased cold acclimation could improve freeze tolerance of turfgrasses and other crops, but the rate and level of cold acclimation primarily affected by temperature are also influenced by factors such as light intensity, day length, cultural practices, and other abiotic stresses such as drought and salinity. Turfgrasses possess various adaptive mechanisms for surviving freezing temperatures, such as increases in certain sugars or amino acids, synthesis of novel proteins, and the degree of instauration of membrane lipid fatty acids. Cold stresses for turfgrasses include winter month N management for both cool- and warm-season turfgrasses. When a soil is colder than optimum temperatures for root activities, N uptake is significantly reduced and the demand for N is to the minimum and plant growth ceases. However, turfgrasses are perennial crops and winter green color is ideal. Several reports stated that late-season N improved fall and spring color of bermudagrass and had little effect on total nonstructural carbohydrate (TNC) levels without any negative effects on post-dormancy recovery in the spring. On the other hand, aggressive late fall season N applications to turfgrasses may increase disease potentials including spring dead spot for bermudagrass, gray snow molds for creeping bentgrasses, and winter kill potential for warm-season turfgrasses.

By observations for two winters, foliar and lower rate N enhanced turf quality of overseeded rough bluegrass on bermudagrasses than 100% granular N fertilizers. Warm-season turfgrasses possess various N-rich defensive metabolites and enzymes to cope with low-temperature stress including some proteins. "Midiron" bermudagrass during cold acclimation was correlated with its superior freezing tolerance compared to "Tifway" in protein contents, and cultivars with greater stolon proline content exhibited greater freezing tolerance than those with less proline during the winter. Zhang et al. (2006a,b) compared "Riviera" (cold tolerant) and "Princess-77" (cold sensitive) bermudagrasses selected and either subjected to cold acclimation at 8°C/4°C (day/night) with a light intensity of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  over a 10 h photoperiod for 21 days or maintained at 25°C/23°C (day/night) with natural sunlight in a glasshouse. Cold acclimation induced accumulation of sugars, proline, and TNC in both cultivars, but protein accumulation was found only in "Riviera" and not in "Princess-77." Superoxide dismutase (SOD) increased during the first 7 days and then declined, while catalase (CAT) and ascorbate peroxidase (APX) activity decreased in response to cold acclimation in both cultivars. Significant correlations of LT50 with sugars, proline, protein, CAT, and APX were obtained in "Riviera," but only with proline and the antioxidant enzymes in "Princess-77." These results suggest screening cold-tolerant cultivars among bermudagrasses with rapid accumulation of C- and N-rich compounds during cold acclimation is possible. However, the directly N nutrient impacts were not included in these reports and future research is needed. Numerous reports have stated that unsaturated membrane lipids play a significant role in both chilling and freezing tolerance with little effects or no effects with saturated fatty acids. There are no direct evidences of N nutrient levels in relation to saturation and instauration of membrane lipids; even transgenic plants have further approved the importance of unsaturated membrane lipids.

### 39.4.7 DROUGHT AND WATERLOGGING STRESSES

Due to the intensive management practices of putting greens, drought and waterlogging situations are normally temporary stresses, which may last hours before the situation is improved. Any types of putting greens may not survive a hot summer day without any irrigation, and daily irrigation is essential for putting greens during hot summer months. However, waterlogging situations may last longer depending on natural precipitations. The unhealthy relationships between water and nutrient for putting green turfgrasses can be summarized as the followings due to improper nutrient management or restrictions:

- Drought with excessive nutrient input
- Drought under nutrient deficiency
- Waterlogging with excessive nutrient input
- Waterlogging under nutrient deficiency

If these situations exist along with temperature extremes and salinity stress, extended drought or waterlogging periods can be detrimental to putting green turfgrasses. Nutrient availability and uptake by roots are affected under these unfavorable water conditions with other factors. The general practice to enhance the nutrient use under water stresses is to protect root systems and minimize damages. Light and frequent fertilization is recommended under drought conditions.

When a putting green is under water stress, reduced fertilizer input can alleviate drought stress and excessive nutrient input will worsen the situation due to increased osmotic stresses in soils. Turfgrasses under drought stresses still need nutrient input but in a reduced rate. The degree of reduction of nutrient rate will depend on the severity of drought, turfgrass used, and other environmental conditions. Drought periods can last from hours to months even in yearly or multiple-year cycles, but these longer periods of drought may affect other types of turf more than putting greens and as mentioned above, daily irrigation is needed during hot summer months. Any approaches to reduce drought stress to putting green turfgrasses will be favorable for nutrient use efficiency, and nutrients like N, K, and Ca have been reported. Bian et al. (2009) reported that with TE treatment to “L-93” creeping bentgrass, during the later phase of 28 days of drought stress, TE-treated plants had increased accumulation of soluble sugars and inorganic ions (Ca and K) in leaves. McCullough et al. (2007) reported TE application can reduce N input for both “L-92” creeping bentgrass and “Tifeagle” bermudagrass putting greens in a transition zone location indicating the potential of controlled N use combining with plant growth regulators for both cool-season and warm-season putting green turfgrasses although there is a shortage of reports on other cool-season and seashore paspalum and zoysiagrass putting greens.

Waterlogging may cause double damages to putting green turfgrasses by losing root functions to effectively absorb nutrients and leaching losses of nutrients from the soils. For nitrogen losses, denitrification can be more significant under waterlogging conditions during warmer days than cooler days. During the waterlogging period, any nutrient applications to soils seem helpless, and the critical time is post-waterlogging management with proper nutrient supply to replace the nutrient lost during the waterlogging stress. Extended waterlogging periods more likely result in nutrient deficiency particularly N deficiency in addition to the root damages, and these damages vary among creeping bentgrass cultivars (Jiang and Wang, 2006; Wang and Jiang, 2007). Due to the weak root systems immediately following a waterlogging period, light and foliar N application is encouraged with caution of hot temperatures.

### 39.4.8 WEAR, BALL MARK, DIVOTS, THATCH LAYER, AND ORGANIC MATTER STRESSES

Excessive thatch layer and organic matter content in putting green soils have negative effects on the soil profile such as reductions in hydraulic conductivity, decreased water infiltration, increased localized dry spots, reduced tolerance to temperature extremes, increased disease and pest problems, and



reduced pesticide and fertilizer effectiveness (Beard, 1973, 2002; Turgeon, 2008; McCarty, 2011). Although there is lack of documents on the relationship between nutrients and thatch and organic matter control for putting greens, it is believed that excessive N input plays negative roles in thatch and organic matter accumulation for both cool-season and warm-season turfgrasses (Engel and Alderfer, 1967; Carrow et al., 1987, 2001; McCarty 2005, 2007). In addition to N, other excessive nutrient input to putting greens may have negative impacts either on thatch accumulation or thatch decomposition such as K, P, and Ca (Callahan et al., 1998). Ledebouer and Skogley (1967) reported that a field study on 15-year-old velvet bentgrass putting turf, sucrose treatments decreased turf quality and modified the stimulating effects of fertilizers. Lime had no influence on turf quality. Sucrose tended to increase the incidence of dollar spot disease, while fertilizer decreased it. They found that the physical thatch structure showed leaf sheaths were more resistant to decay than clippings or sloughed leaves. Nodes and crown tissues were most resistant to decomposition. To date, mixed findings on thatch and organic matter content control of both cool-season and warm-season turfgrass putting greens still exist related to control methods such as coring, vertical mowing, and others; frequency of coring and others; topdressing practices; and chemical thatch control methods. However, it has been agreed that coring and vertical mowing are the two effective ways to remove thatch or organic matter from putting green root zones and that vertical mowing has less interference with playability (Fu et al., 2009; Rowland et al., 2009; McCarty, 2011).

All types of turf require cultural and cultivation practices to improve soil compaction, reduce thatch accumulation, and recover from wear, ball marks, and divots for sports and golf course turf use. The frequency, intensity, and type of cultivation heavily depend on the turf use, turfgrass species and cultivar, and the environmental conditions. Golf course putting greens, tees, and fairways require at least once a year of hollow tine core cultivation (in most cases multiple cultivations per year) plus other types of cultivation to improve the soil and root zone conditions and reduce thatch accumulation (Beard, 2002; McCarty, 2011). Sports fields and lawns are also recommended cultivation once per year. Nitrogen and other nutrients play a significant role to help turfgrass recover from the temporary disruption caused by cultivation. Turfgrass cultivation includes hollow tines, solid tines, slicers, groomers, hydrojet (high-pressure water beams vertically penetrating turf soil profile), and vertical cutting. These processes temporarily stress the turf, and proper nutrient supplies help the turf recovery. The soil cultivation with opening holes provides better contacts between fertilizers and roots and controlled-release N fertilizers plus other nutrients are normally recommended. However, if cultivation is conducted under stresses, more caution is needed with proper nutrient supply. Light and frequent nutrient applications in many such cases help the turf grow evenly. Proper nutrient application can minimize the time requirement to resume the playability for golf courses and sports fields. Nutrient deficiencies, particularly with N, slow the recovery from cultivation disturbance or damages.

The positive effects of plant nutrients on turfgrass wear tolerance and recovery have been reported (Trenholm et al., 2001; Hoffman et al., 2010a,b) and there is a lack of reports on putting green wear studies in general, particularly turf maintenance mechanic practices associated wear and traffic stresses.

#### 39.4.9 INSECT AND MITE PEST STRESSES

Insect and mites require high N dietary for their need for amino acids and proteins. Normally high N contents in plants benefit insect and mite pests. High N fertility levels result in rapid growth and succulent tissues that may also increase the chance of damages from insect and mite pests. Excessive N fertilization associated with improper mowing, watering, and fungicide application encourage accumulation of thatch layer in some turfgrasses. A thick layer of thatch is a good habitat for chinch bugs (*Blissus* spp.), billbugs (*Sphenophorus* spp.), sod webworms (*Crambus* spp.), two-lined spittlebug [*Prosapia bicincta* (Say)], and several other insects and mites. Heavy N fertilization to turfgrasses stimulates rapid leaf and shoot growth with the costs of root growth and with the

relatively reduced root growth and development. Under such a situation, the turfgrass will have to overcome more severe stress when the environmental conditions are not favorable for roots, such as the summer time for cool-season turfgrasses and cool spring time for warm-season turfgrasses. In addition, the turfgrasses with poor root systems will suffer more severe damages from subsurface feeders. On the other hand, a slightly higher N supply may encourage foliar growth and speedy recovery from foliar feeders' damages.

Endophyte infections with some turfgrasses including *Fescue*, *Lolium* species provide one of the most important natural resistance to surface feeders, and maybe subsurface feeders, and N deficiency to the host turfgrasses can discourage the symbiotic relationship since most of the alkaloids produced between the endophyte and hosts are N-dependent secondary N metabolites. There is a lack of information on N form influences in turf insect and mite pests including earth worms as pests. However, recent reports indicate the black turfgrass ataenius [*Ataenius spretulus* (Haldeman)] density was encouraged by using N fertilizers as sewage sludge but with no effects when using manure-rich N fertilizers. Turfgrass species with resistance to turf insect and mites vary at both the species and cultivar levels, and the resistances include speedy recovery which is highly related to the efficiency of nutrient acquisition and production of the N-related metabolites.

Multiple factor ecosystems including host N status, pest, and natural enemy are complicated and interesting since plant N status can affect insect consumption rates and population dynamics of herbivorous insects and mites. The changes of such ecosystems rather heavily depend on carbon deposit in addition to the plant N statuses. For a turfgrass ecosystem, multiple factors are involved, including consistent disturbance of mowing and the traffic of insect and mite pests.

Development of insect resistance turfgrass species or cultivars is promising since diversified resistances exist among turfgrass species to insect and mite pests. The resistant turfgrass species or cultivars need sufficient N supply in order to produce enough toxins, which are most likely the case for the insect and mite resistance, and unhealthy turfgrasses either due to N deficiency or excessiveness will significantly lose the resistance.

## 39.5 SELECTED NUTRIENTS WITH IMPACTS TO PUTTING GREEN TURFGRASSES UNDER STRESSES

Putting green management is different than traditional crop systems, and the relative importance of nutrient management putting greens will depend on turfgrass species and cultivars, locations, and the maintenance levels. However, the general common characteristic associated with nutrient management is the challenge of sandy soils with relatively low cation exchange capacity (CEC) and frequent cycles of water saturation and dryness. Nutrient toxicities of putting greens are rarely reported unless the continuous use of recycling water. The nutrient management for putting greens under stresses has not been fully investigated and many areas remaining unclear. In order to have the nutrients organized in Sections 39.5.1 through 39.5.3, these nutrients are grouped according to the frequency of the stress problems associated observed by the authors. The nutrients excluded in this section are purely due to the length limitation of the chapter and the authors are intentionally to update the two beneficial nutrients of silicon and sodium.

### 39.5.1 THE GROUP WITH THE GREATEST IMPACTS

#### 39.5.1.1 Nitrogen

Nitrogen is the most needed nutrient in quantity for all green plants and crops including turfgrasses, and it has been most actively investigated among all turf nutrients due to N's significant roles for turf growth, color, quality, and use. Nitrogen is a constituent of almost any compounds in plants except carbohydrates. These compounds include proteins, chlorophyll, hormones, nucleic acids, and secondary metabolites. Under N deficiency, turf stresses can be worsened and N plays the most

significant role among all nutrients to recover turf under stresses (Liu et al., 2008a,b). On the other hand, excessive N input can increase turf stress severity including less resistance to extremes of environmental conditions for growth and higher potential damages from pests. Excessive N very often may be even worse than slight N deficiency to manage turf under stresses regardless of N loss potential from turf–soil systems.

Putting greens require the highest amount of N input than any other type of turfgrass, and it is mainly due to the frequent removal of clippings. Despite the impossible solution of returning clippings for putting greens, the challenging research for alternative solutions to save nutrient input to putting greens is needed.

### 39.5.1.2 Potassium

Potassium (K) is an essential macronutrient for turfgrasses and the high demand of growing putting green turfgrasses for K is generally recognized by golf course superintendents. Potash ( $K_2O$ ) fertilizers are often applied to putting greens to maintain the turf quality, and as a matter of fact K has been applied as much as N to putting greens (Throssell et al., 2009). However, even for a well-fertilized putting green, K deficiency can frequently occur due to dominant sandy soils of putting greens with lower CEC and high potential of leaching. With increased costs of fertilizers, nutrient usage efficiency of putting greens has become more important for turfgrass nutrient management.

Under salinity stress, K-use efficiency for putting green turfgrasses can be critical and Na/K ratios play significant roles in many crops for salinity tolerance. However, there is lack of information on specific recommendations of K for putting green turfgrasses with poor water quality and salinity stresses. An average of 200–300 kg ha<sup>-1</sup> K has been applied to putting greens annually and there still is room for further K efficiency improvement although root zone K toxicities are rarely reported. Liquid and foliar K applications to putting greens require cautions to keep from fertilizer burning, which is often associated with drought and heat stresses.

Under other stressful conditions, K supply is critical in many aspects for turfgrasses. Regardless of the positive impacts of proper K input on stresses such as cold, salinity, drought, and diseases, K input to a cool-season or warm-season turfgrass putting green is different by rates and application methods. Significant discrepancies of K effects related to stresses are found in the literature and a part of the reasons may be variable study conditions used and with different turfgrasses in addition to a lack of uniformity of control of stressful or favorable factors in the soils, media, and environment. However, as mentioned, direct K toxicities to plants have rarely been reported, and the research of K interactions with other nutrients under stressful conditions for putting greens has not fully started yet.

### 39.5.1.3 Phosphorus

In plants, the most important function of P is to activate both enzymes and metabolic intermediates and provide reversible energy storage in ATP. P is also a major structural component of nucleic acids and membrane lipids and takes part in regulatory pathways involving phospholipid-derived signaling molecules (e.g., phosphatidylinositol and inositol triphosphate) or phosphorylation reactions (e.g., MAP kinase cascades) (Marschner, 1995; Taiz and Zeiger, 2006).

Putting greens with sand as the dominant soil texture often require regular and routine P applications. Guertal (2006) stated that P fertilization of sand-based greens should not be neglected, and slightly higher rates or more frequent application than what have been recommended by current soil-test recommendations may be warranted after a two-year period of P nutrient use and uptake study on bermudagrass putting greens. Although there is a lack of specific research on the relationship between P and stress reduction, a recent report shows that root distribution of creeping bentgrass could be manipulated by spatial localization of P supply in the root zone with buffered P sources.

Putting green P application and the interaction of P with soil microbial activities including symbiotic associations with mycorrhizae are the important aspects to enhance P use efficiency particularly under stresses.

#### 39.5.1.4 Iron

The relationship of Fe with other turf stresses (Table 39.2) has not been intensively investigated yet, and enhancement of Fe uptake and use by turfgrasses under stressful conditions can provide bright future to meet the purpose of minimized input with maximized outcome since Fe is one of the turf nutrients that can change turf color and appearance within hours. The relationship of Fe with other nutrients and heavy metals and the antagonism with P for turfgrass management deserve more attention. Recently, Fe interactions with herbicides used for turf weed control have been investigated, and contradicted results have been reported (Massey et al., 2006; McCullough and Hart, 2009).

There is about 5% Fe by weight of the earth's crust, and Fe is the fourth most abundant element next only to oxygen, silicon, and aluminum in most soils. For turfgrasses, Fe is one of the micronutrients with dramatic effects to turf color and quality and it may be the most commonly used micronutrients for turf management particularly for sand-based soils and golf putting greens, which often have a high potential of Fe deficiency with lower soil CEC. Soils are normally well furnished with Fe, but in well-aerated and in calcareous soils Fe is found in oxide and hydroxide compounds with a very low solubility. So, putting green turfgrasses often have to face an iron deficiency in sandy soils and frequent Fe applications to putting greens are recommended. Most turfgrasses contain a normal range of Fe within 200 mg kg<sup>-1</sup> with a normal range of 50–200 mg kg<sup>-1</sup> in green tissues by dry weight. As a structural component of cytochrome, hemes, hematin, ferriochrome, and leghemoglobin, the essential compounds for oxidation–reduction reactions in both photosynthesis and respiration Fe is essential for turf color, growth, and appearance.

Iron is absorbed by roots as inorganic forms of Fe<sup>2+</sup> and Fe<sup>3+</sup>, with Fe<sup>2+</sup> as the dominant form being taken up by plants after it is reduced from Fe<sup>3+</sup> in the rhizosphere. The activity of Fe<sup>3+</sup> in the soil solution is highly pH dependent and its activity decreases 1000 fold for each pH unit rise when the soil pH is above neutral. Under most soil conditions, grasses have the capability to excrete iron chelators, called phytosiderophores, from the roots to solubilize the external insoluble Fe<sup>3+</sup>. The amount of these phytosiderophores increases under Fe deficiency stress.

When turfgrass tissue Fe content is less than 50 mg kg<sup>-1</sup>, Fe deficiency is likely to occur and it may happen more often in cool-season turfgrasses since most cool-season turfgrasses contain higher Fe in tissues. Iron deficiency symptoms appear in younger leaves first because the immobility of Fe in plants is very limited. Iron deficiency may occur under several soil stressful conditions. Soil Fe availability is also affected by soil moisture, temperature, organic matter content, and the balance with other micronutrients or heavy metals. Excessive cation nutrients such as Cu, Mn, and Zn plus toxic elements such as Cd in soil solutions can inhibit Fe uptake and use by turfgrasses.

Fe applications to deficient grasses can improve the uptake of N and other elements (Marschner, 1995). Foliar Fe application of ferrous sulfate and of chelated Fe to turfgrass can correct Fe deficiency effectively. Detailed information on inorganic Fe and chelate Fe sources on turf is listed in Carrow et al. (2001). Summer-heat-induced Fe chlorosis can occur during the high-temperature stress periods of midsummer for both cool-season and warm-season turfgrasses. This condition often occurs on a number of cool-season grasses grown on both sand and on clay-loam field soils. Devetter et al. (2008) found that higher application rates in the range of 1.12 kg Fe ha<sup>-1</sup> were required at the time of the onset of chlorosis to overcome the problem.

Under acidic and wet soil conditions, excessive Fe can become toxic with consequences of Mn deficiency and form black layers under the turf root zone when Fe reacts with excessive soil sulfur under anaerobic conditions. Black layers often occur in anaerobic soil conditions. Anaerobic bacteria produce hydrogen sulfide gas, which is poisonous to turfgrass roots. Hydrogen sulfide also reacts chemically with metal elements such as Fe, creating black deposits, which form black layers within the soil.

## 39.5.2 THE IMPORTANT GROUP

### 39.5.2.1 Sulfur

Plants require sulfur (S) as a nutrient because S is required to synthesize the S-containing amino acids cystine, cysteine, and methionine. The main structural function of S in proteins is to form disulfide ( $-S-S-$ ) bonds, which help proteins form third- and fourth-level structures. Sulfur is also needed to form coenzyme A, which is important for the oxidation of fatty acids in the citric acid cycle and for chlorophyll formation. In addition, S is involved in chlorophyll stabilization for green plants (Marschner, 1995).

Grasses normally contain 0.10%–0.50% S by dry weight. Sulfur is actively absorbed as sulfate ( $SO_4^{2-}$ ); however, very small amounts of  $SO_2$  or other sulfur compounds can be absorbed by leaves or roots as gaseous forms (Carrow et al., 2001). It is common for sulfate to compete with other anions for plant uptake in the soil solution. The interactions of  $SO_4^{2-}$  with soil minerals are affected by the Fe/Al ratio, clay content, and soil pH.

The visual symptoms of S deficiencies resemble N, Fe, and Mg deficiencies with yellowish, chlorotic condition of the leaves. Since S is not as mobile as N in plants, younger leaves can show S deficiency symptoms first. Relatively small amounts of S are needed by plants in relation to N. Although soil organic matter provides a better pool of available S than N, high N applications can cause turfgrass S deficiencies. This is particularly true in sandy soils subjected to frequent leaching (Carrow et al., 2001).

Sulfur deficiency is often observed with salt-affected sandy soils. Sulfur deficiencies for turfgrasses may not occur frequently because of the use of S-containing fertilizers and the capability of  $SO_2$  absorption by turfgrass leaves. Turfgrass can also receive S from rain water. The use of high sulfur coal in industry can add large amounts of S to the atmosphere. Killorn (1983) reported that the central part of the United States receives an estimated 13.5–16.8 kg ha<sup>-1</sup> per year in rainfall.

However, frequent removal of clippings can cause S depletion from some soils, since leaves on average contain about 0.2% to 0.6% S by dry weight (Carrow et al., 2001).

High  $SO_2$  concentrations in the atmosphere can be toxic to plants in some industrial areas by production of sulfuric acid ( $H_2SO_4$ ) on the turfgrass leaves. These acids are destructive to cuticle layers of plant leaves. Direct S toxicity by plant excessive S uptake is not a problem and the excessive S can be released to the atmosphere as  $H_2S$  from leaves (Carrow et al., 2001). Frequent use of sulfur-coated urea and sulfate-containing fertilizers, including gypsum ( $CaSO_4$ ), can add S to turfgrasses. Acidic soil conditions and high S contents in soils can suppress some patch diseases such as take-all patch, summer patch, and spring dead spots (Dernoeden and O'Neil, 1983; Carrow et al., 2001; Vargas, 2005).

Sulfur can be found under anaerobic conditions associated with excessive Fe to form black layers in soils (Hodges, 1992; Carrow et al., 2001). Black layers often occur in anaerobic soil conditions created by compaction and/or over irrigation. The anaerobic conditions cause sulfur-reducing bacteria to use sulfate or elemental sulfur instead of oxygen during the respiration process, which produces hydrogen sulfide ( $H_2S$ ) gas, which is poisonous to turfgrass roots. The hydrogen sulfide reacts with metal elements such as Fe, creating black deposits of FeS, which form black layers within the soil and inhibit turfgrass growth.

The use of sulfuric acid to treat irrigation water is a common practice where carbonate ( $CO_3^{2-}$ ) and bicarbonate ( $HCO_3^-$ ) levels are high and excess sodium (Na) exists. Bicarbonates can react with Ca and Mg in the water and can remove them from solution. This reduction of soluble Ca and Mg results in an increase in the sodium adsorption ratio (SAR) of the water, which increases the likelihood of soil structure problems caused by Na deflocculating clay particles (Christians, 1999). The process of adding sulfuric acid to irrigation water requires a thorough evaluation of the soil conditions and water chemistry before it is initiated.

### 39.5.2.2 Calcium

Calcium (Ca) is the fifth most abundant element in the earth's crust with an average soil calcium concentration of 3.6%. Calcium is passively absorbed as  $Ca^{2+}$ . After being absorbed,  $Ca^{2+}$  is

preferentially attracted into the intercellular spaces and into the root cortex. Calcium deficiencies and toxicities are rarely reported for putting green turfgrasses. This is partially due to the relatively low requirement turfgrasses for Ca and the abundance of Ca in the putting green root zones. The concentration of Ca in turfgrasses ranges from 0.3% to 1.25% of dry matter (Carrow et al., 2001) which varies among the various turfgrass species and even within cultivars of the same species. Irrigated turfgrass can receive certain amounts of Ca from the irrigation water and rainfall typically has 8 mg L<sup>-1</sup> Ca whereas irrigation water frequently has 25–50 mg L<sup>-1</sup> Ca (Carrow et al., 2001).

When deficiencies occur, symptoms are seen on newer leaves, leaf tips, and leaf margins, since Ca is relatively immobile in the plant. Since Ca is essential for cell elongation and division, the new leaf tips and margins are often distorted. The leaf tips and margins may have a reddish-brown color, and may wither and die (Carrow et al., 2001). Calcium deficiencies can also reduce root elongation, mucilage production, and root cell division, creating shortened and stunted roots (Carrow et al., 2001).

Many soils that are low in Ca have high concentrations of soluble Al, Mg, Mn, H, and/or Na, which can out-compete Ca for the cation exchange sites in the soil and in the intercellular apoplasm of roots. Calcium deficiency symptoms are rarely seen on the shoots of plants grown on these types of soils. However, roots from plants grown in these types of soils are often stunted, thin, and spindly and are brownish/black in color. Adding calcium to soils with high levels of Al, Mn, H, or Na usually improves turfgrass growth and development due to the improvement of the damaged root systems.

True Ca phytotoxicity in turfgrasses has not been reported yet, and putting greens may be stressed by soluble salt fertilizer burn when high rates of CaCl<sub>2</sub> or CaNO<sub>3</sub> are applied particularly under drought and heat stresses. Frequent applications of Ca can also reduce the levels of Mg and K found in turfgrass leaves or on cation exchange sites (St. John et al., 2003). Putting green sandy soils often have a lower CEC and become more easily affected by competition among cations causing reductions in availability for uptake.

### 39.5.3 THE BENEFICIAL GROUP

This is an interesting group of nutrients, and there are more than two belonging to this group. The selected two, Si and Na, may be even more interesting or beneficial for putting green management currently or for the future in addition to every active research of these two's positive roles for plants and crops in the recent year.

#### 39.5.3.1 Silicon

Silicon has been studied over 150 years for its roles in living things, and in the past two decades, it has rapidly raised interests for crops including turfgrass management (Table 39.3). Some turf managers have routinely applied silicon forms to their turf as nutrient supply and protection of abiotic and biotic stresses regardless whether it is defined as essential or not for plants (Hull, 2004; Datnoff, 2005). The significance and updated research of Si in turfgrasses have been recognized by a couple of relatively new reviews specifically focusing on turfgrasses.

Silicon is the second most abundant mineral element in soils next to oxygen, which comprises 38% of the earth's crust, while Si comprises approximately 31% of the earth's crust by weight. Although Si is about 4 times and 6 times of the third and fourth most abundant elements, Al and Fe, of 7% and 5% of the earth's crust by weight, respectively, neither mineral O nor Si fortunately is any major stressful factor to living things on the earth. In contrast, gaseous oxygen is essential for all living things except groups of anaerobic microorganisms, and to date mineral forms of O have not been identified with serious direct impacts to plants.

Despite the abundance of Si in soils, Si deficiency can still occur due to the high demand and Si depletion from continuous planting of crops such as rice. Silicon deficiency mainly occurs in upland rice production fields with low soil pH and in highly weathered soils. Silicon has not been classified as an essential mineral nutrient for plants yet, but most grasses including turfgrasses on average

contain 1% of their dry weight as Si. This is just less than N and K tissue concentrations and some textbooks even list Si as macronutrients. Although a standard or recommended Si concentration in any turfgrass species are not available yet, Si applications to turfgrasses increasing turfgrass tissue Si concentrations with an active uptake and enhancing wear tolerance and disease resistance were found in different turfgrass species.

Silicon often occurs as insoluble minerals in the earth's crust, and soil solutions on average contain less than 20 mg soluble Si L<sup>-1</sup>, which is still several time folds of soluble P. Soluble Si in soils is actively taken up by plant roots as silicic acid, Si(OH)<sub>4</sub>. Some species in the grass family, such as wetland rice, can accumulate as much as 10%–15% of their dry shoot weight as Si, and some broadleaf plants can reach 3% Si in dry shoot and leaf weight. This is much higher than the most demanded N, P, and K concentrations in these plants. Silicic acid is transported in plants from roots to leaves mainly through the xylem (Marschner, 1995) and Si is either to be deposited underneath cuticle layer to protect plants from pathogens or stay in cell walls to strengthen plants. Silicic acid concentrations inside plant roots can be many times that of the Si levels in the soil nutrient solution, and an active root Si uptake happens dominantly. Recently, root plasma membrane Si transporters have been identified in rice, which are responsible for root membranes' Si influx and efflux. This type of membrane transporters are also found in corn and barley with unique features being only localized at the root endodermis with no polarity.

As an essential (rice) or beneficial nutrient (many other plants), Si has been identified to play physiological roles to strengthen cell wall structure, enhance lignin biosynthesis, interact with other essential nutrients, and reduce toxicity of some heavy metals including Al, Mn, and Fe. Silicon has been shown to interact with other macro- and micronutrients, in particular to strengthen compartmentations of micronutrients in plants and even uptake. Silicon deficiency for rice mainly occurs in upland rice production fields with low soil pH and long history of crop production and in highly weathered soils.

Enhanced disease resistance in response to Si applications in combination with other nutrients has been reported for both warm- and cool-season turfgrasses. Tremblay et al. reported that under both greenhouse and field conditions, Si applications controlled dollar spot on creeping bentgrass. Brecht et al. (2004) reported a 30% gray leaf spot control on St. Augustinegrass by Si applications. Recently, Brecht et al. identified the controlling effects of silicon fertilization on pathogenicity of *Bipolaris cynodontis* and *Curvularia lunata* on Floradwarf bermudagrass. With further understanding the turfgrass disease resistance enhancement by Si, nutrient management including Si may have a potential to target more serious putting green diseases caused by pathogens of *Pythium*, *Rhizotonia*, and *Typhula*.

Trenholm et al. (2001) found a 20% reduced wear injury following the foliar application of potassium silicate at 1.1 kg and 2.2 kg Si ha<sup>-1</sup> and applied as a soil drench at 22.4 kg Si ha<sup>-1</sup> to seashore paspalum putting greens. However, significant positive benefits of Si on turfgrass insect resistance have not been fully identified even though it would be expected that high Si concentration in plant tissue would play a role in suppression of insect feeding. With the effect of lignin biosynthesis enhancement, application of Si may negatively influence forage crops' quality, and the specific Si effects on putting green thatch accumulation have not been identified yet.

There is very limited information on Si deficiency or toxicities in turfgrasses, and there is no information on symptoms of Si deficiency and toxicity among turfgrasses. The difficulty of accurate analysis of soil and plant tissue Si concentrations has not been fully resolved by using glass tubes for Si digestion from soil and plant tissues. Further investigations of the direct, indirect, physiological, pest resistance enhancement, and practical benefits of Si to turfgrass species and cultivars and golf putting green management are needed.

### 39.5.3.2 Sodium

The earth's crust has 2.8% Na and 2.6% K by weight, but the earth is a salty globe with majority of water as ocean water containing on average 30 g NaCl L<sup>-1</sup>. Sodium is an essential element for animals and humans and Na must be present in relatively large amounts in their diet. Sodium

is the principal electrolyte in animal and human systems to maintain the ionic balance of body tissues and fluids. The osmotic characteristics of Na are utilized in the blood stream to regulate osmotic pressure within the cells and body fluids in order to avoid excessive loss of water. Plants use K as the principal electrolyte even with plenty of Na in the soils due to the total different water use and regulation mechanisms between plants and animals. Sodium can substitute K particularly with C<sub>4</sub> plants, but plants still exhibit a strong preference for K (Marschner, 1995; Subbarao et al., 2003).

Sodium is not an essential plant nutrient, but it may be worthy to respect it from plant nutritional point of view or as an interesting element for putting green turfgrass management for the future. For cool-season putting green turfgrass management, except in certain degree of pest suppression of Na effects, excessive Na is toxic to C<sub>3</sub> plants. However, Na's possible essential-nutrition role in warm-season turfgrasses particularly for salinity-tolerant species deserves more attention (Munshaw et al., 2004). Although Na has never been reported being deficient for putting green turfgrasses, it is often found that turfgrass tissue dry weight contains about 10–100 mg kg<sup>-1</sup> of Na when turfgrasses are grown in sandy soils without salinity stress.

Halophytes are defined as any plant, especially a seed plant, that is able to grow in habitats excessively rich in salts. These plants have special physiological adaptations that enable them to absorb water from soils and from seawater, which have solute concentrations that non-halophytes could not tolerate. Some halophytes are actually succulent, with a high water-storage capacity.

## 39.6 SUMMARY AND PROSPECTS

With new challenges of limited resources, turfgrass management becomes more and more limited for options with resources but with more options with integrated management and approaches. Putting greens, as one of the most intensively managed artificial monoculture crop systems, the functions and economic values are unique and will evolve. ISM will not only bring the future turf managers for multiple-dimensional approaches but also enhance the readiness for local, regional, even international management challenges. The functions and demands of function crops such as turfgrasses can meet with the new demands and it does not always mean being the highest expenses and prettiest appearance, rather the majority of the people using the turf are better served. Nutrient management for turfgrasses have been one of the most active research areas in turfgrass science and management since the turfgrass research started more than a century ago and it will continue with the ultimate goal: “use less and make the best out of it.”

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## *Part VIII*

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*Climatic Changes, Elevated Carbon  
Dioxide, and Plant/Crop Responses*

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# 40 Plant Biomass and Stem Juice of the C<sub>4</sub> Sugarcane at Elevated Growth CO<sub>2</sub> and Temperature

*Joseph C.V. Vu and Leon H. Allen Jr.*

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## 40.1 INTRODUCTION

The global demand for agricultural crops for human food and livestock feed is rising at a tremendous pace. With rapid increases in world population, fossil fuel consumption, industrial development, and deforestation, atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]), currently at about 385 μmol mol<sup>-1</sup>, has been projected to surpass 700 μmol mol<sup>-1</sup> before the end of this century (Solomon et al., 2007). Consequently, a rise in [CO<sub>2</sub>] and other greenhouse gases would cause severe impacts on future global climate, including an increase in air temperature (Schneider, 2001; Solomon et al., 2007) and an alteration in the rainfall patterns in many areas of the world (Schneider, 2001; Long et al., 2004). The increases in ambient [CO<sub>2</sub>] and temperature are particularly of major concern when considering their interactive effects on future agricultural crop production, as well as the distribution of terrestrial ecosystems, including natural forests and grasslands. As competition for available cultivable land and fresh water resources for agriculture will undoubtedly escalate, increasing the efficiency in productivity for economically important crops under future climate change scenarios to satisfy greater worldwide demand for human food and livestock feed is a critical challenge.

A rise in atmospheric [CO<sub>2</sub>] by itself could stimulate photosynthesis and enhance the growth and the productivity of agricultural crops. Particularly, for many C<sub>3</sub> plant species, the present atmospheric [CO<sub>2</sub>] limits their photosynthetic capability, and a higher atmospheric [CO<sub>2</sub>] reduces photorespiration and enhances leaf photosynthetic CO<sub>2</sub> exchange rate (CER), thus increasing plant growth and yield (Bowes, 1993). For C<sub>4</sub> plants, because of their unique foliar Kranz anatomy feature and associated cellular physiology, they are able to concentrate [CO<sub>2</sub>] at the site of ribulose biphosphate carboxylase-oxygenase to levels many times higher than ambient [CO<sub>2</sub>]. The photosynthesis of C<sub>4</sub> plants is practically near saturation at current atmospheric [CO<sub>2</sub>], and a rise in ambient [CO<sub>2</sub>] may presumably induce little or no enhancement on their growth and yield. Nevertheless, a positive growth response to elevated growth [CO<sub>2</sub>] has been reported for a variety of C<sub>4</sub> species, although



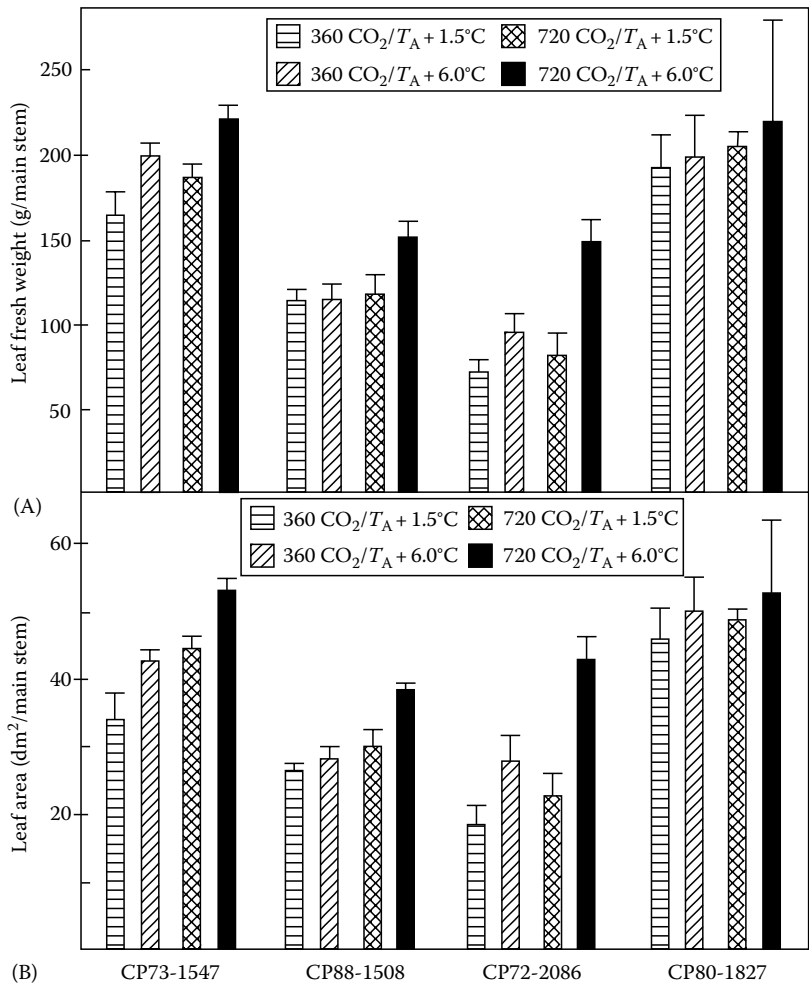
to a smaller extent when compared with  $C_3$  plants (Kimball, 1993; Poorter, 1993; Ziska and Bunce, 1997; Maroco et al., 1999).

$C_4$  plants represent less than 4% of all terrestrial angiosperm species; however, their economical impact is substantial (Brown et al., 2005). On a global basis, up to one-third of agricultural crop productivity is provided by  $C_4$  plants (Cerling et al., 1997; Ghannoum et al., 1997; Brown et al., 2005). Maize, millet, sorghum, and sugarcane are the most economically important  $C_4$  monocots in terms of human food production (Brown, 1999; De Souza et al., 2008; Edgerton, 2009). Particularly for sugarcane, up to 75% of the world sugar production is provided by this crop species (Glassop et al., 2007; De Souza et al., 2008; Edgerton, 2009). In addition to being the major source of sugar production for human consumption, sugarcane biomass has been used for livestock feed in many regions of the world. With the recent international fossil fuel crisis, many nations have been forced to consider other alternative sources of energy to reduce their high-cost dependency on fossil oil imports, and sugarcane and the cereal grain maize have become the central crops for biofuel production (Goldemberg, 2007; Glassop et al., 2007; Edgerton, 2009).

This contribution presents part of the results from our studies with sugarcane that were carried out in Gainesville, Florida (29°38'N and 82°22'W). Our primary focus will be on plant biomass, stem juice, and stem sugar of this  $C_4$  crop in response to elevated growth  $CO_2$  and temperature, with a discussion directed toward improvement in sugarcane productivity to meet future global needs for food and bioenergy. Four cultivars of sugarcane (*Saccharum officinarum* L.), namely CP73-1547 (cultivar 1), CP88-1508 (cultivar 2), CP72-2086 (cultivar 3), and CP80-1827 (cultivar 4), were first propagated in February 1997 from stalk cuttings in a greenhouse at 30°C. They were then grown from mid-March to June in paired-companion, temperature-gradient greenhouses (TGGs) in galvanized metal containers, 1.5-m long  $\times$  0.6-m wide  $\times$  0.6-m deep, containing organic soil. The structural characteristics, the specific methods, and the quality of  $[CO_2]$ , and the temperature controls and the growth conditions for sugarcane in these TGGs were previously described in detail (Vu et al., 2002, 2006; Vu and Allen, 2009). In summary, each TGG was a free-standing unit consisting of a semicylindrical, arch-shaped structure, 29.3-m long, 4.3-m wide and 2.2-m high at the ridgepole, which was made of galvanized steel and covered with a transparent polyethylene plastic that transmitted 90% of solar photosynthetic photon flux density so that plants received direct, nearly natural solar irradiance. Each TGG was divided into a 3.6-m long entry section to stabilize the incoming flow (north end), four sequential experimental segments, each 5.5-m long, and a 1.8-m flow convergence zone, before the air was expelled by a computer-controlled, variable speed ventilation fan mounted at the south end. This ventilation fan controlled the air flow and regulated the temperature gradient continuously 24 h/day, which averaged from 1.5°C above outside ambient temperature ( $T_A$ ) at the air-entry at the north end (segment 1, near-ambient temperature) to 6.0°C above  $T_A$  at the south end (segment 4, high temperature). The daytime  $[CO_2]$  was maintained in one TGG at an ambient concentration ( $\sim 360 \mu\text{mol mol}^{-1}$ ) by air flowing in directly from outside, and at a double-ambient (elevated) concentration ( $\sim 720 \mu\text{mol mol}^{-1}$ ) in the other by injecting  $CO_2$  provided from a refrigerated reservoir located outside the TGG. Soil moisture was checked daily and additional irrigation water was applied, as needed, to ensure adequate soil moisture for plant growth. Fertilizers containing macro and micro nutrients were applied biweekly during the growth season at recommended doses for commercial sugarcane production in Florida (Obreza et al., 1998). Minimum/maximum air temperatures outside the TGGs were 9.7°C/23.2°C, 14.2°C/27.4°C, 12.0°C/25.9°C, 16.4°C/29.7°C, and 19.7°C/30.7°C for the months of February, March, April, May and June, respectively. In late June, test plants were harvested for determinations of biomass and stem sugar.

## 40.2 LEAF BIOMASS AND AREA

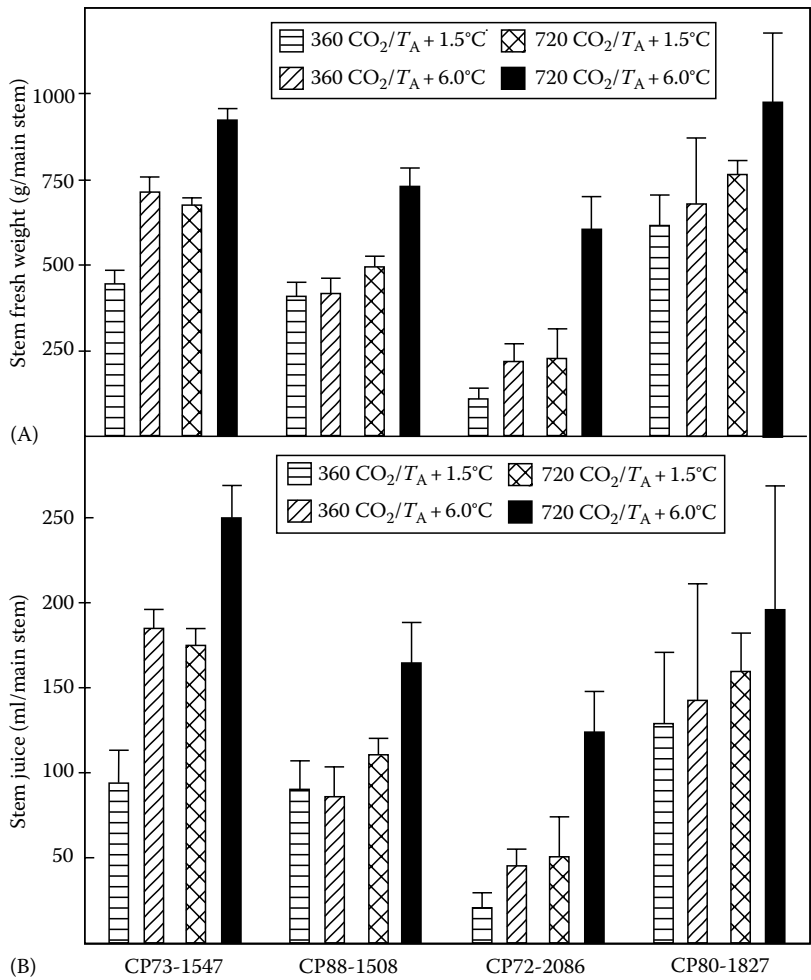
For sugarcane grown at doubled  $[CO_2]$ /high temperature, the increases in the total leaf fresh weight per main stem were 34%, 33%, 108%, and 15% for cultivars 1, 2, 3, and 4, respectively, compared with plants grown at ambient  $[CO_2]$ /near-ambient temperature (Figure 40.1A). Although the



**FIGURE 40.1** Total leaf fresh weight (A) and area (B) per main stem of four cultivars of sugarcane grown for 3 months at daytime CO<sub>2</sub> concentrations of 360 (ambient) and 720 (double-ambient) μmol mol<sup>-1</sup> and under temperatures of 1.5°C and 6.0°C higher than the outside ambient temperature (T<sub>A</sub>). Values are the mean and SE of four determinations for the cultivars CP73-1547, CP88-1508, and CP72-2086, and of two determinations for the cultivar CP80-1827.

enhancements in leaf biomass by doubled [CO<sub>2</sub>] or high temperature alone were less compared with the doubled [CO<sub>2</sub>]/high temperature combination, they were still noticeable (Figure 40.2). In terms of [CO<sub>2</sub>] effects, doubled [CO<sub>2</sub>] plants at the near-ambient temperature, compared with ambient [CO<sub>2</sub>] plants grown at a similar temperature, had increases up to 16% (cultivar 3) in the leaf fresh weight. Similarly, at high temperature, double-ambient [CO<sub>2</sub>] enhanced the leaf fresh weight by 11% (cultivar 1) to 57% (cultivar 3), when compared with plants at ambient [CO<sub>2</sub>]. With respect to the temperature effects, ambient [CO<sub>2</sub>] plants grown at high temperature were up to 33% (cultivar 3) greater in leaf fresh weight than their ambient [CO<sub>2</sub>] counterpart plants grown at a near-ambient temperature. Similarly, doubled [CO<sub>2</sub>] plants grown at high temperature, compared with doubled [CO<sub>2</sub>]/near-ambient temperature plants, had the leaf fresh weight increased up to 80% (cultivar 3).

The total leaf area per main stem for the four cultivars of sugarcane was enhanced at double-ambient [CO<sub>2</sub>] and high temperature (Figure 40.1B). In terms of [CO<sub>2</sub>] effects, double-ambient [CO<sub>2</sub>] enhanced the leaf area by 31%, 13%, 22%, and 6% at near-ambient temperature and 25%, 36%, 54%, and 6% at high temperature for cultivars 1, 2, 3, and 4, respectively. In terms of temperature effects,



**FIGURE 40.2** Main stem fresh weight (A) and total juice volume per main stem (B) of four cultivars of sugarcane grown for 3 months at daytime CO<sub>2</sub> concentrations of 360 (ambient) and 720 (double-ambient)  $\mu\text{mol mol}^{-1}$  and under temperatures of 1.5°C and 6.0°C higher than the outside ambient temperature ( $T_A$ ). Values are the mean and the SE of four determinations for the cultivars CP73-1547, CP88-1508, and CP72-2086, and of two determinations for the cultivar CP80-1827.

high temperature increased the leaf area by 25%, 6%, 51%, and 9% for the ambient [CO<sub>2</sub>] plants, and 20%, 28%, 90%, and 9% for the doubled [CO<sub>2</sub>] plants for cultivars 1, 2, 3, and 4, respectively. Such increases were even greater for plants grown under a combination of doubled [CO<sub>2</sub>]/high temperature. The leaf area of plants grown under doubled [CO<sub>2</sub>]/high temperature, when compared with plants grown at ambient [CO<sub>2</sub>]/near-ambient temperature, was enhanced 56%, 45%, 132%, and 15% for cultivars 1, 2, 3, and 4, respectively.

### 40.3 STEM BIOMASS, JUICE, AND SUGAR

The fresh weights of the main stem for the four cultivars of sugarcane grown at two [CO<sub>2</sub>] and temperatures are shown in Figure 40.2A. When compared with plants grown at ambient [CO<sub>2</sub>]/near-ambient temperature, plants grown at doubled [CO<sub>2</sub>]/high temperature were 113%, 78%, 468%, and 48% greater in stem fresh weight, respectively, for cultivars 1, 2, 3, and 4. In terms of temperature effects, ambient [CO<sub>2</sub>] plants grown at high temperature were up to 106% (cultivar 3) more in stem

fresh weight. Similarly, doubled [CO<sub>2</sub>] plants grown at high temperature were 37%, 47%, 173%, and 19% greater in stem fresh weight for cultivars 1, 2, 3, and 4, respectively, when compared with doubled [CO<sub>2</sub>] plants grown at a near-ambient temperature. In terms of [CO<sub>2</sub>] effects, doubled [CO<sub>2</sub>] plants at a near-ambient temperature, compared with ambient [CO<sub>2</sub>] plants grown at a similar temperature, had 56%, 21%, 108%, and 24% more in stem fresh weight for cultivars 1, 2, 3, and 4, respectively.

Sugarcane juice production per main stem was enhanced substantially by growth at doubled [CO<sub>2</sub>] and high temperature (Figure 40.2B). Plants grown at doubled [CO<sub>2</sub>]/high temperature combination, when compared with plants grown at ambient [CO<sub>2</sub>]/near-ambient temperature combination, had stem juice increased by 165%, 82%, 493%, and 52% for cultivars 1, 2, 3, and 4, respectively. Growth at high temperature alone had stem juice increased up to 118% (cultivar 3) for the ambient [CO<sub>2</sub>] plants, and up to 143% (cultivar 3) for the doubled [CO<sub>2</sub>] plants. The increases in stem juice for plants grown at doubled [CO<sub>2</sub>], when compared with plants grown at ambient [CO<sub>2</sub>], were 83%, 23%, 144%, and 23% at the near-ambient temperature, and 36%, 91%, 172%, and 38% at high temperature, for cultivars 1, 2, 3, and 4, respectively.

Sugarcane stem juice consists of a high concentration of water-soluble sugars (primarily sucrose) (Moore, 1995). Although the percent of total soluble solids in the stem juice (BRIX) extracted from cultivars 1 and 2 was not appreciably affected by growth at doubled [CO<sub>2</sub>], a high temperature might enhance it (13%–32% for cultivar 1 and 9%–19% for cultivar 2) (Table 40.1). The stem juice BRIX of both ambient and doubled-ambient [CO<sub>2</sub>] plants averaged 9% for cultivar 1, and 7.5% for cultivar 2, for growth at both the near-ambient temperature and at high temperature. The total amount of soluble solids per main stem, computed based on the values of stem juice BRIX and of the whole stem juice volume (Figure 40.2B), shows that plants grown at doubled [CO<sub>2</sub>]/high temperature were up to threefold greater in main stem soluble solids than those grown at ambient [CO<sub>2</sub>]/near-ambient temperature. By plotting the sugarcane stem sucrose concentrations as a function of the internode position, using the values of 106, 483, 571, 608, and 556 mM respectively for internode 2 (immature, expanding internode at plant top), 10, 20, 30, and 40 (mature, most developed internode located at plant base) as reported by Moore (1995), the amount of sucrose for an individual plant (g/main stem)

**TABLE 40.1**  
**BRIX, Total Soluble Solids, and Sucrose of Stem Juice of the Two Cultivars of Sugarcane, CP73-1547 and CP88-1508, Grown at Ambient (360 ppm) and Double-Ambient (720 ppm) [CO<sub>2</sub>], and Temperatures of 1.5°C and 6.0°C above Ambient Temperature (*T<sub>A</sub>*)**

| Parameters                                        | 360 μmol mol <sup>-1</sup> CO <sub>2</sub> |                              | 720 μmol mol <sup>-1</sup> CO <sub>2</sub> |                              |
|---------------------------------------------------|--------------------------------------------|------------------------------|--------------------------------------------|------------------------------|
|                                                   | <i>T<sub>A</sub></i> + 1.5°C               | <i>T<sub>A</sub></i> + 6.0°C | <i>T<sub>A</sub></i> + 1.5°C               | <i>T<sub>A</sub></i> + 6.0°C |
| CP73-1547                                         |                                            |                              |                                            |                              |
| BRIX (%) (w/v)                                    | 8.6 (0.1) a                                | 9.7 (0.9) a                  | 7.8 (0.4) a                                | 10.4 (1.9) a                 |
| Soluble solids (g main stem <sup>-1</sup> )       | 8.2 (0.3) d                                | 17.9 (1.3) b                 | 13.6 (0.7) c                               | 26.1 (2.1) a                 |
| Sucrose (g main stem <sup>-1</sup> ) <sup>a</sup> | 11.4 (0.5) c                               | 22.1 (1.6) b                 | 20.9 (1.1) b                               | 30.1 (2.4) a                 |
| CP 88-1508                                        |                                            |                              |                                            |                              |
| BRIX (%) (w/v)                                    | 7.6 (1.4) a                                | 8.3 (1.3) a                  | 7.0 (0.3) a                                | 8.3 (1.1) a                  |
| Soluble solids (g main stem <sup>-1</sup> )       | 6.9 (1.2) b                                | 7.2 (1.1) b                  | 7.8 (0.2) b                                | 13.6 (1.0) a                 |
| Sucrose (g main stem <sup>-1</sup> ) <sup>a</sup> | 10.8 (1.5) b                               | 10.3 (1.4) b                 | 13.4 (1.4) b                               | 19.8 (1.7) a                 |

*Note:* Values are the means and SE (parentheses). Values within rows with the same letter indicate no significant difference at a 5% level in a Duncan multiple range test.

<sup>a</sup> Sucrose was estimated by assuming that the stem internode sucrose concentration was 350 mM, based on values as reported by Moore (1995).

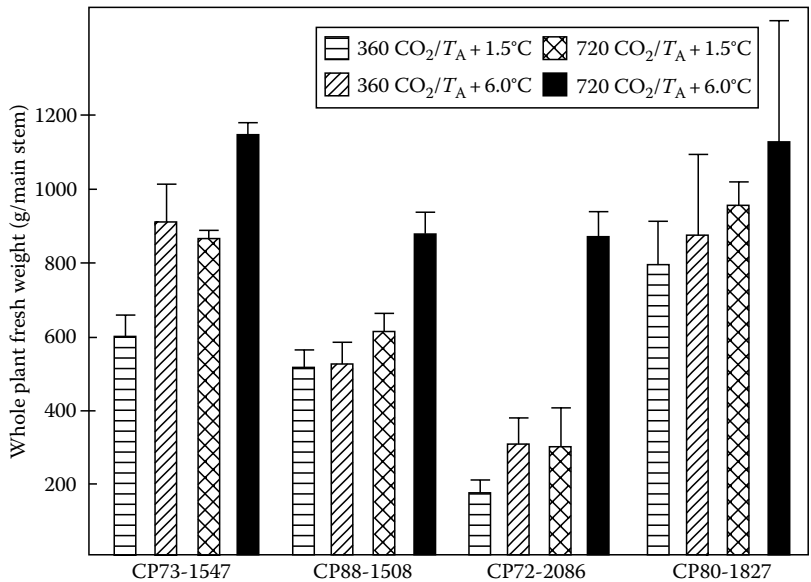
can be estimated. Our test plants were about 4 months old with main stems averaging 10 internodes at the time of harvest. The stem sucrose concentration for both cultivars 1 and 2 was close to 350 mM for all CO<sub>2</sub>/temperature treatments, and plants grown at a double-ambient [CO<sub>2</sub>]/high temperature could have up to 2.5-fold higher amounts of sucrose per main stem than those grown at ambient [CO<sub>2</sub>]/near-ambient temperature (Table 40.1).

40.4 ABOVEGROUND PLANT BIOMASS

The total aboveground plant (leaf + stem) fresh weights per main stem for the four sugarcane cultivars grown at the [CO<sub>2</sub>] and the temperature treatments are shown in Figure 40.3. Plants grown at the doubled [CO<sub>2</sub>]/high temperature, when compared with those at the ambient [CO<sub>2</sub>]/near-ambient temperature, were 91%, 68%, 324%, and 40% greater in total fresh weight for cultivars 1, 2, 3, and 4, respectively. In terms of [CO<sub>2</sub>] effects, plants grown at doubled [CO<sub>2</sub>]/near-ambient temperature, when compared with those at ambient [CO<sub>2</sub>]/near-ambient temperature, had the total fresh weight increased by 44%, 17%, 73%, and 20% for cultivars 1, 2, 3, and 4, respectively. In terms of temperature effects, ambient [CO<sub>2</sub>] plants grown at high temperature were up to 76% (cultivar 3) greater in total fresh weight, when compared with ambient [CO<sub>2</sub>] plants grown at a near-ambient temperature. Similarly, doubled [CO<sub>2</sub>] plants grown at high temperature were up to 145% (cultivar 3) greater in total fresh weight than doubled [CO<sub>2</sub>] plants grown at a near-ambient temperature.

40.5 OVERALL RESPONSE OF SUGARCANE TO ELEVATED [CO<sub>2</sub>] AND TEMPERATURE

Table 40.2 shows the average percent increases by double-ambient [CO<sub>2</sub>] and high temperature for various plant components of the four sugarcane cultivars. The percent increases were computed from the data pooled across the four cultivars as presented in Figures 40.1 through 40.3, and their averages are reported. Across the four cultivars and [CO<sub>2</sub>]/temperature treatments, the average percent increases for



**FIGURE 40.3** Whole plant (leaf + stem) fresh weight of four cultivars of sugarcane grown for 3 months at daytime CO<sub>2</sub> concentrations of 360 (ambient) and 720 (double-ambient) μmol mol<sup>-1</sup> and under temperatures of 1.5°C and 6.0°C higher than the outside ambient temperature (T<sub>A</sub>). Values are the mean and SE of four determinations for the cultivars CP73-1547, CP88-1508, and CP72-2086, and of two determinations for the cultivar CP80-1827.

**TABLE 40.2**  
**Average Percent Increase for Various Growth Parameters of Four Cultivars of Sugarcane in Response to Double-Ambient [CO<sub>2</sub>] and High Temperature**

| Plant Components      | Double-Ambient [CO <sub>2</sub> ] Effect                  |                                           | High Temperature Effect                 |                                         |
|-----------------------|-----------------------------------------------------------|-------------------------------------------|-----------------------------------------|-----------------------------------------|
|                       | <i>T<sub>A</sub></i> + 1.5°C <sup>a</sup><br>(% Increase) | <i>T<sub>A</sub></i> + 6.0°C <sup>b</sup> | Ambient [CO <sub>2</sub> ] <sup>c</sup> | Doubled [CO <sub>2</sub> ] <sup>d</sup> |
| Leaf area             | 17.9 (2.7) b                                              | 30.1 (5.0) a                              | 22.9 (5.1) ab                           | 36.7 (9.1) a                            |
| Leaf fresh wt.        | 9.6 (1.5) b                                               | 27.4 (5.4) a                              | 14.6 (3.8) b                            | 33.6 (7.9) a                            |
| Juice volume          | 68.6 (14.5) a                                             | 84.2 (15.9) a                             | 54.5 (15.1) a                           | 64.6 (13.3) a                           |
| Stem fresh wt.        | 53.0 (10.4) a                                             | 79.3 (17.0) a                             | 45.2 (12.1) a                           | 68.2 (17.0) a                           |
| Total plant fresh wt. | 38.5 (5.5) b                                              | 65.5 (13.3) a                             | 34.5 (8.9) b                            | 59.7 (11.5) a                           |

*Note:* The percent increases were computed and averaged from the data pooled across the four sugarcane cultivars presented in Figures 40.1 through 40.3. Values are the means and SE (parentheses). Values within rows with the same letter indicate no significant difference at a 5% level in a Duncan multiple range test.

<sup>a-d</sup> Percent increases for various plant components at each specific [CO<sub>2</sub>]-temperature treatment are computed as follows:

<sup>a</sup> [(Data at doubled CO<sub>2</sub>/*T<sub>A</sub>* + 1.5°C)/(data at ambient CO<sub>2</sub>/*T<sub>A</sub>* + 1.5°C)] × 100.

<sup>b</sup> [(Data at doubled CO<sub>2</sub>/*T<sub>A</sub>* + 6.0°C)/(data at ambient CO<sub>2</sub>/*T<sub>A</sub>* + 6.0°C)] × 100.

<sup>c</sup> [(Data at ambient CO<sub>2</sub>/*T<sub>A</sub>* + 6.0°C)/(data at ambient CO<sub>2</sub>/*T<sub>A</sub>* + 1.5°C)] × 100.

<sup>d</sup> [(Data at doubled CO<sub>2</sub>/*T<sub>A</sub>* + 6.0°C)/(data at doubled CO<sub>2</sub>/*T<sub>A</sub>* + 1.5°C)] × 100.

the various growth parameters were 18%–37% for leaf area, 10%–34% for leaf fresh weight, 45%–79% for stem fresh weight, 55%–84% for stem juice, and 35%–66% for total plant fresh weight. The average percent increases for most components were greater for plants grown at the doubled [CO<sub>2</sub>]/high temperature combination than plants grown at doubled [CO<sub>2</sub>]/near-ambient temperature or ambient [CO<sub>2</sub>]/high temperature combination.

## 40.6 PERSPECTIVES AND CHALLENGES

The four sugarcane cultivars grown for 3 months either at double-ambient [CO<sub>2</sub>] or high temperature, or under double-ambient [CO<sub>2</sub>]/high temperature combination, had greater stem juice production than their counterparts grown at ambient [CO<sub>2</sub>]/near-ambient temperature combination. In addition, the sugarcane plants grown at double-ambient [CO<sub>2</sub>]/high temperature had greater leaf area, and leaf and stem fresh weights than those grown at ambient [CO<sub>2</sub>]/near-ambient temperature. The increases in leaf area and plant biomass were also greater for plants grown at double-ambient [CO<sub>2</sub>]/high temperature than those grown either at elevated [CO<sub>2</sub>] or high temperature alone. A recent report by De Souza et al. (2008) also showed that plants of the sugarcane cultivar SP80-3280 grown at 720 μmol mol<sup>-1</sup> [CO<sub>2</sub>] had greater total biomass by the 13th week and accumulated 25%, 60%, and 40% more in leaf, stem, and total biomass after 50 weeks than plants grown at 370 μmol mol<sup>-1</sup> (ambient) [CO<sub>2</sub>]. The positive response of the well-watered and adequately fertilized sugarcane plants to elevated [CO<sub>2</sub>] of this study and those reported by De Souza et al. (2008) is in contrast to the two NADP-ME C<sub>4</sub> type monocots maize and sorghum, which only respond to elevated growth [CO<sub>2</sub>] when soil moisture becomes limited (Ottman et al., 2001; Kim et al., 2006; Leakey et al., 2006). A model has been recently developed to predict the potential yield of sugarcane for the regions of Sao Paulo (Brazil) and Queensland (Australia) under future climate change scenarios (Da Silva et al., 2008). This model, which simulates the impacts of increased air [CO<sub>2</sub>] and temperature on plant growth, shows a yield increase of 12%–16% for sugarcane under doubled atmospheric [CO<sub>2</sub>] and a moderate increased air temperature of up to 2.7°C. However, other important

parameters including soil water availability and plant cultivar should also be integrated in a future simulation model for sugarcane in response to rising atmospheric  $[\text{CO}_2]$  and temperature.

The enhancement in sugarcane plant total leaf area at elevated growth  $[\text{CO}_2]$  and temperature is of particular interest. Recently, a number of cell wall-related genes have been found responding to elevated growth  $[\text{CO}_2]$  for this NADP-ME  $\text{C}_4$  type monocot (De Souza et al., 2008). Xyloglucan endotransglycoxylase/hydrolase (XTH), which is involved in cell wall expansion and cell enlargement, is up-regulated 3.6-fold in sugarcane leaves after 13 weeks of plant growth at double-ambient  $[\text{CO}_2]$ . Such stimulation in the XTH transcript abundance occurs with a concomitant increase of 145% in leaf area (De Souza et al., 2008). An increase in whole plant leaf area per se would enhance the ongoing and cumulative photosynthetic capability of the entire plant under elevated  $\text{CO}_2$  and/or high temperature, even though the photosynthetic CER of fully expanded mature leaves are less stimulated by elevated  $[\text{CO}_2]$  when compared with young developing leaves (De Souza et al., 2008; Vu and Allen, 2009). The increase in whole plant leaf area, together with an improvement in leaf/plant water-use efficiency (Vu and Allen, 2009), may partially explain the enhancement in plant biomass and stimulation in juice production and soluble solids concentration of the stem for sugarcane grown at elevated  $[\text{CO}_2]$ . In sugarcane, the stem is made up of internodal segments with different stages of development along the stem. Sucrose, which is synthesized in the leaf, is translocated to the stem where it is stored (Moore, 1995; Lingle, 1999). Since sucrose concentration starts to increase in the internodes as elongation ceases, young expanding internodes at the plant top have relatively small BRIX accumulation rate ( $<10\%$ ) and low sucrose concentration ( $\sim 100\text{ mM}$ ), whereas fully developed mature internodes located lower down the stem have stem juice BRIX up to threefold higher and stem sucrose up to sixfold greater (Welbaum and Meinzer, 1990; Moore, 1995; Rae et al., 2005). In sugarcane, sucrose accounts for about 70% of the stem juice soluble solids in the lower internodes and can exceed 90% in fully mature internodes (Moore, 1995). A cell wall invertase has been recently identified to be down-regulated in leaves of sugarcane under elevated growth  $[\text{CO}_2]$  (De Souza et al., 2008). Since activity of the invertase has been suggested to regulate the export rate of carbohydrates from the source leaves to the sink organs (Foyer, 1987), the lower expression of the invertase in sugarcane leaves would consequently explain the higher accumulation of sucrose in the stem under elevated growth  $[\text{CO}_2]$  (De Souza et al., 2008).

The global demand for agricultural crops for bioenergy, in addition to food for human and feed for livestock, is accelerating rapidly (Edgerton, 2009). Exhaustible fossil fuels, which at present make up about 80% of the global energy supply, cannot be considered as the world's main source of energy for more than one or two future generations (Goldemberg, 2007). New renewable energy sources to secure a reliable, constant, and sustainable global energy supply for the future must be prioritized. Maize, sugarcane, sugar beet, sorghum, wheat, and nonfood crops, including switch grass and other vegetative sources, are all suitable for the fulfillment of this purpose. At the present time, maize in the United States and sugarcane in Brazil account for about 80% of the global ethanol in use (Goldemberg, 2007). The United States is currently the world leading nation using grains, primarily maize, and to a lesser extent, wheat, for biofuel production. Such grain-based biofuel is expected to grow from  $\sim 31$  billion liters for the year 2008 to  $\sim 56$  billion liters in 2017 (Edgerton, 2009). For sugarcane, Brazil is leading in the cultivation of this crop to make ethanol that is used as a partial substitute for fossil gasoline. The Brazilian sugarcane ethanol industry, which started in the 1970s, is today highly competitive with fossil gasoline and is increasing fast, with production rising from 18 billion liters in 2006/2007 to 22.2 billion liters in the 2007/2008 (Goldemberg, 2007; Goldemberg et al., 2008; Zuurbier and van de Vooren, 2008). With worldwide continued increase in demand for food and bioenergy, an identification of the sugarcane cultivars carrying superior traits in water-use efficiency, leaf photosynthetic performance, and stem sugar yield under future rises in  $[\text{CO}_2]$  and changes in climate need to be explored. Consequently, improvements in sugarcane stem sucrose and plant biomass through both conventional and new breeding methodologies should be carried out at a high priority. Similarly, an identification of cultivars with superior traits and an improvement in yield with new breeding methodologies are also necessary for maize

and other crops. The introduction of biotechnology traits and the development of new breeding technology using DNA-based markers, in combination with continued advances in agricultural system improvements and agronomic practices, could double the yield of maize in the United States, currently at about 10 ton ha<sup>-1</sup>, by the year 2030 (Edgerton, 2009). Improving the productivity of sugarcane, maize, and other important agricultural crops, together with the development of and the widespread use of new farming technologies, would allow growers to supply future global needs for food and energy in a sustainable fashion while minimizing the conversion of large areas of new land into crop production (Edgerton, 2009). However, the production capacities of the plants and the availability of resources including light, water, and nitrogen, not human demand, will in reality determine how much plant biomass can be grown for feedstock, for ethanol, and how much biofuel the world can produce (Sinclair, 2009).

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## *Part IX*

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*Future Promises: Improving Plant  
and Crop Adaptation/Tolerance and  
Cultivation under Stressful Conditions*

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# 41 Improving Crop Resistance to Abiotic Stresses through Seed Invigoration

*Muhammad Farooq, Abdul Wahid, Shahzad M.A. Basra, and Kadambot H.M. Siddique*

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## 41.1 INTRODUCTION

Environmental stresses such as drought, salinity, nutrient excess and deficiency, temperature extremes, and submergence are major limitations to harvest potential crop yield globally. Lesser than 10% arable land of the world may be free from abiotic stresses, with salinity and drought the most pervasive (Zhu 2002).

Drought is a consistent feature across much of the 63.5 Mha sown annually, most of which lies in tropical Asia, Africa, and Latin America (Narciso and Hossain 2002). Of 40 Mha grown under rainfed lowlands in Asia, about 15 Mha are often affected by flooding and submergence (Huke and Huke 1997). Potential yield losses by individual abiotic stresses are estimated to be 17% by drought, 20% by salinity, 40% by high temperature, 15% by low temperature, and 8% by other factors (Ashraf and Harris 2005). Temperature fluctuations have strong influence, even more than other abiotic stresses, on the crop growth, productivity, and quality (Wahid et al. 2007a). Cold stress, although seasonal, may have a similar effect as that of drought stress; as upon freezing of water, concentrates of cellular solutes increases substantially, thus plants face a state of water deficit.

Owing to intricacy of interactions between stress factors and plant development mechanism (Zhu 2002), tolerance to environmental stresses, at cellular as well as whole plant levels, is a complex phenomenon (Foolad et al. 2003; Farooq et al. 2009a–d). Developing crop plants tolerant of environmental stresses is considered a promising strategy, which may help meeting the growing food needs of developing and underdeveloped countries. Development of stress-tolerant plants thus requires thorough understanding of mechanism involved in stress-induced damages and tolerance thereof. During past two decades, significant advancement in biotechnological research has unraveled the tolerance mechanism to environmental stresses at the molecular

level (Prabhavathi et al. 2002; Farooq et al. 2009a,b,d). Plants' basic cellular responses to environmental stresses are almost similar; nonetheless there may be inter- and intraspecific variations for tolerance mechanisms at different developmental stages (Zhu 2001a,b, 2002; Farooq et al. 2009a,b,d). Likewise, oxidative and osmotic stresses, and protein denaturation, may be observed in plants upon exposure to environmental stresses. All these may lead to adaptive responses, e.g., stress protein expression, accumulation of compatible solutes, and antioxidant system at cellular level (Zhu 2002).

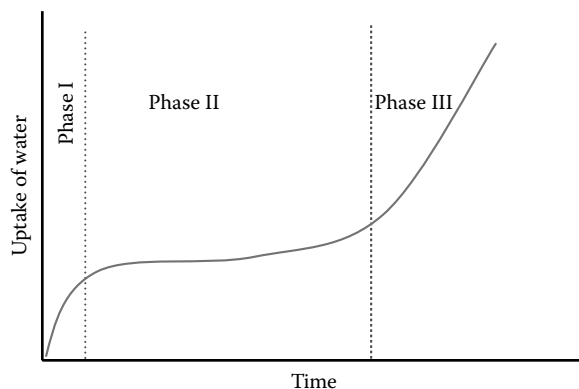
Seed invigoration is a broad term involving several techniques aimed at improving seed delivery and crop performance under a wide range of environmental conditions (Farooq et al. 2009c). Different seed invigoration tools have been used for better establishment of seedlings or better plant development/yield under stressful environments (Basra et al. 2005; Iqbal and Ashraf 2007a,b; Afzal et al. 2008; Farooq et al. 2008a–f, 2009a–g). Several review articles (e.g., Raghothama 1999; Thomashow 1999; Masclaux et al. 2001; Samac and Tesfaye 2003; Shinozaki et al. 2003; Tester and Davenport 2003; Farooq et al. 2009a–d) and textbooks (e.g., Taiz and Zeiger 2006) have discussed the strategies to improve tolerance to abiotic stresses. There are also reviews on the role of pre-sowing seed treatments in abiotic stress tolerance (Ashraf and Foolad 2005) and rice seed invigoration (Farooq et al. 2009c), but this is the first comprehensive review encompassing previous work on improving abiotic stress tolerance using seed invigoration approaches.

## 41.2 SEED INVIGORATION FOR STRESS RESISTANCE

Seed invigoration is a relatively new term to describe techniques and treatments applied on a given seed lot for value addition and improved field performance (Farooq et al. 2009c). Although used interchangeably with seed priming (Farooq et al. 2006c), it also encompasses many pre-sowing seed treatments. Seed invigoration or seed enhancements are “post-harvest treatments” to improve germination and seedling growth or help in the provision of seeds and other materials required at the time of sowing (Taylor et al. 1998). This definition includes four general methods: pre-sowing hydration treatments (Basra et al. 2006; Farooq et al. 2006a–d, 2007a,b), low molecular weight osmoprotectants seed treatments (Taylor et al. 1998), coating technologies (Ross et al. 2000; Song et al. 2005), and pre-sowing thermal treatments (Farooq et al. 2004b, 2005). These treatments focus on shortening the time to seedling emergence and protecting seeds from biotic and abiotic factors during critical phases of seedling establishment. Such treatments synchronize emergence, resulting in uniform and vigorous stands, and improved yield. The role of seed invigoration in crop resistance to abiotic stresses is discussed in the following sections.

### 41.2.1 SEED HYDRATION TREATMENTS

Seeds require moisture, optimum temperature, and oxygen, and sometimes light, for germination. Water uptake follows a tri-phasic pattern (Figure 41.1; Bewley 1997). Phase I is imbibition, which commences with the physical uptake of water by seeds. Imbibition usually occurs rapidly due to the large difference in water potential between dry seeds and water. In living seeds, little metabolic activity occurs during this phase. In fact, dead seeds imbibe water at the same rate as viable seeds. Phase II is the lag period where water uptake is minimal, but considerable metabolic activity is evident. The seed converts stored reserves (proteins, fats, and lipids) into compounds needed for germination. Phase III is radicle protrusion, which usually coincides with radicle emergence and is characterized by rapid water uptake (rapid increase in fresh weight). Phases I and III are controlled by the availability of water to seeds (Taylor et al. 1998). Depending on the nature of hydration method, pre-sowing hydration techniques can be placed in two groups; a brief account of each, with special reference to abiotic stress tolerance, has been given below.



**FIGURE 41.1** Tri-phasic curve of seed germination. During seed priming, seeds are placed in a solution with low water potential, which prevents seeds from imbibing sufficient water required for germination. As a result, the duration of Phase II is extended, which increases the hydrolysis of reserved food material.

#### 41.2.1.1 Presoaking

In presoaking methods, seeds are soaked in simple water with or without aeration (Thornton and Powell 1992). As no osmoticum or salt is added, water imbibition is not controlled. Presoaking has several hydration techniques with potential for seed vigor enhancement: hydropriming (Farooq et al. 2006e), hardening (Farooq et al. 2004a), and on-farm priming (Harris et al. 2000, Harris 2006). Of those, hydropriming has shown promise in improving crop resistance against abiotic stresses.

In hydropriming, seeds are soaked, misted in water, and then re-dried before completing germination, which minimizes the use of chemicals and avoids discarding materials (Soon et al. 2000). In many studies, hydropriming substantially improved crop performance under stress conditions, such as improving the resistance to salinity in wheat (Nath et al. 1991; Aschermann-Koch et al. 1992; Kahlon et al. 1992; Roy and Srivastava 1999; Basra et al. 2005), barley (Kibite and Harker 1991), maize (Bennet and Waters 1987; Ashraf and Rauf 2001), sunflower (Kaya et al. 2006), pigeon pea (Jyotsna and Srivastava 1998), and *Acacia* (Rehman et al. 1998).

In summary, presoaking treatments may improve stress tolerance in crop plants; however, priming duration is vital in this regard. Soaked seeds must be re-dried to safer moisture levels, and can be sown later, if not immediately.

#### 41.2.1.2 Seed Priming

Priming of seed is a controlled hydration technique where seeds are partly hydrated to allow metabolic events to occur without germination, and are then re-dried to permit routine handling (Heydecker and Coolbear 1977; Bradford 1986). To control hydration, seeds are placed in solutions with high osmotic potential. This prevents seeds from entering Phase III of hydration by extending and holding seeds within the lag phase (Phase II) (Figure 41.1; Taylor et al. 1998). As seeds are metabolically active during this period, they convert stored reserves for germination such that membrane and genetic repair is better than under normal imbibition. Seeds are then removed from the priming solution, rinsed with water, and dried. Such seeds germinate faster than non-primed seeds.

Primed seeds usually have higher and synchronized germination (Heydecker and Coolbear 1977; Brocklehurst et al. 1984; Farooq et al. 2009c) owing to simply a reduction in the lag time of imbibition taking place (Heydecker 1977; Bewley and Black 1982; Bray 1995; Taylor et al. 1998; Welbaum et al. 1998; McDonald 2000; Brocklehurst and Dearman 2008), build up of germination-enhancing metabolites (Farooq et al. 2006d), metabolic repair during imbibition (Burgass and Powell 1984; Bray et al. 1989), and osmotic adjustment (Bradford 1986). Seed priming can be accomplished by different means as detailed below.

#### 41.2.1.2.1 Osmopriming

During osmoconditioning or osmopriming, to control water imbibition, seeds are soaked in aerated low water potential solutions of polyethylene glycol (PEG), glycerol, sorbitol, or mannitol, and prevent radicle protrusion (Bray 1995); seeds are then re-dried close to their original weight. Several studies have proved that osmopriming improved germination in a wide range of crops, especially under adverse conditions (Heydecker et al. 1975; Brocklehurst et al. 1984; Bradford 1986; Karssen et al. 1989; Bradford and Haigh 1994; Brocklehurst and Dearman 2008). When planted in the field, primed seeds usually germinate quickly and evenly under stressful conditions. For example, osmopriming substantially improved salinity resistance in tomato (*Lycopersicon esculentum*) and asparagus (*Asparagus officinalis*) (Pill et al. 1991), stand establishment in cucumber (*Cucumis sativus*) when primed with mannitol (Passam and Kakouriotis 1994), germination and stand establishment in Bermuda grass (*Cynodon dactylon*) under salt stress (Al-Humaid 2002), and germination and early seedling growth in asparagus under drought stress with  $-1.0$  MPa PEG (Bittencourt et al. 2004).

Osmopriming not only improves seed germination and stand establishment but also enhances general crop performance under optimal and stressful conditions. For example, osmopriming (with PEG 6000) of canola (*Brassica napus*) seeds significantly improved crop performance under both normal and salinity stress (Ehsanfar et al. 2006). Likewise, osmopriming of Italian ryegrass (*Lolium multiflorum*) and sorghum (*Sorghum bicolor*) seeds improved germination rate, seedling growth, and dry matter production under water-stressed, waterlogged, cold-stressed, and saline conditions (Hur 1991). Improved salinity resistance of crop plants with primed seeds has been stemmed from improved osmotic adjustment, as there was significant improvement in sugars and organic acids in leaves and more  $\text{Na}^+$  and  $\text{Cl}^-$  in roots from primed seeds than plants from non-primed seeds (Cayuela et al. 1996).

Available evidence suggests that osmopriming improves crop resistance to chilling stress (Farooq et al. 2008c,d). He et al. (2002) reported more and quicker germination of osmoprimed rice seeds under low temperature ( $5^\circ\text{C}$ ) stress. Likewise, Posmyk et al. (2001) observed that osmopriming may mitigate chilling-induced injuries in soybean (*Glycine max* (L.) Merr.). There was a sharp decline in *in vivo* ACC-dependent ethylene production upon exposure to chilling stress in untreated soybean seeds, while substantial increase in electrolyte leakage indicating membrane deterioration was also observed. Nonetheless, none of these events occurred in primed seeds placed at  $1^\circ\text{C}$ . Moreover, chilling treatments longer than 6 days induced malondialdehyde (MDA) accumulation in the embryonic axis in control seeds than osmoprimed seeds (Posmyk et al. 2001).

Osmopriming (with mannitol) and hydropriming substantially improved the chickpea seedling growth under drought stress (Kaur et al. 2002). Sucrose phosphate synthase (SPS), amylase, sucrose synthase (SS), and invertases (acid and alkaline) activities in shoots were also substantially improved by seed priming. In addition, there was a two-fold increase in specific activity of SPS in cotyledons of primed seedlings (Kaur et al. 2002). In summary, osmopriming may improve seedling establishment and crop performance under stress conditions.

#### 41.2.1.2.2 Halopriming

In halopriming, seeds are soaked in aerated, low water potential solutions of inorganic salts to control water uptake and prevent protrusion of radicle; seeds are then re-dried close to the original weight. A number of studies have shown significant improvement in seed germination, seedling emergence, and establishment, and final crop yield by halopriming under stress. Henckel and Strogonov (1961) in a foundation experiment showed that salinity tolerance may be improved by seed treatment with different salts before sowing, as plants from treated seeds perform better in saline soils than the untreated control.

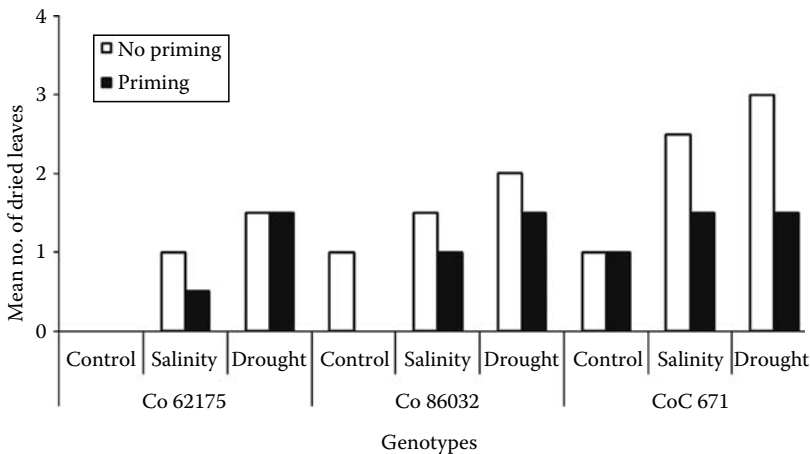
Rice direct seeding is often a common practice in upland rainfed areas; in their experiment, Du and Tuong (2002) evaluated the performance of direct-seeded rice by seed priming with different osmotica. Seed priming with KCl and saturated  $\text{CaHPO}_4$  was the best to improve stand establishment, phenology, and grain yield. Several other studies found that halopriming with mixed salts improved

germination, stand establishment, and early seedling growth under saline conditions in rice (Chang-Zheng et al. 2002; He et al. 2002; Ruan et al. 2003). Recently, Patanè et al. (2009) documented that halopriming improved stand establishment in sweet sorghum (*Sorghum bicolor* cv. Roce).

There was substantial improvement in emergence and stand establishment, when wheat seeds treated with different soaking and drying-cycles were sown in saline soil (Idris and Aslam 1975). Priming with NaCl substantially improved total emergence and dry weight under salt stress in melon seed (Sivritepe et al. 2003). Likewise, halopriming of *Echinacea purpurea* seed with 0.1%  $\text{MnSO}_4$  or 0.05%  $\text{ZnSO}_4$  solution increased germination by 36% and 38% and field emergence by 27% and 41%, respectively (Babaeva et al. 1999). Patade et al. (2009) evaluated the role of salt priming in improving sprouting and plantlet growth in contrasting sugarcane cultivars and reported a significant improvement in both percentage and rate of sprouting in the tolerant (Co 62175) and moderately tolerant (CoM 265) varieties than the sensitive (CoC 671) and test variety (Co 86032) by halopriming with NaCl (100 mM). Likewise, halopriming substantially reduced leaf senescence in sugarcane under drought and salinity stresses (Figure 41.2), although the extent of senescence was similar in both stresses when priming treatments were not applied (Figure 41.2; Patade et al. 2009).

Seed priming with  $\text{CaCl}_2$  and KCl improved chilling tolerance in different plant species such as wheat (Farooq et al. 2008a) and maize (Farooq et al. 2008d,e). Osmopriming of maize seeds with different K salts accelerated germination under chilling stress (Basra et al. 1988). Seeds primed in solutions of either 2.5%  $\text{K}_2\text{HPO}_4$  or 2.5%  $\text{K}_2\text{HPO}_4 + \text{KNO}_3$  improved chilling tolerance compared with untreated seeds, and the effect was largely retained after the seeds were dried. Embryo phospholipid fractions and sterols increased during salt priming, and the proportion of phospholipid diphosphotidyl glycerol also increased. This increase in diphosphotidyl glycerol content was suggested to be due to enhanced internal organization of mitochondrial membranes, while the benefit of osmopriming might have been partly due to greater potential for ATP accumulation (Basra et al. 1988). Seed priming with  $\text{KH}_2\text{PO}_4 + \text{KNO}_3$  significantly increased germination rate, percentage, and synchronization at 10°C (Nerson and Govers 1986). In a classical study, priming in salts and PEG solutions improved the germination at low temperatures in Sugar Baby watermelon; the effect was retained in dried seeds for at least 20 weeks (Sachs 1977).

Among other studies, osmopriming of canola (with  $\text{KNO}_3$ ) improved germination percentage of primed seeds. Radicle length, seedling height, dry weight, and leaf number of plants from primed seeds were also higher compared with unprimed seeds (Hassanpouraghdam et al. 2009). Ashraf and Ruaf (2001), while investigating distilled water or NaCl, KCl,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  halopriming of seeds, reported that though seed priming with all the salt agents was effective in mitigating the adversities



**FIGURE 41.2** Effect of halopriming on leaf senescence in terms of leaf drying in sugarcane plants after 15 days of salinity or drought stress. (Modified from Patade, V. Y. et al., *Agric. Ecosyst. Environ.*, 134, 24, 2009.)

of salinity on maize during germination, priming with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was the most effective with improved germination and seedling growth.

There was significant improvement in dry matter accumulation, net photosynthesis, and grain yield under salinity stress by seed priming with  $\text{CaCl}_2$  followed by KCl and NaCl. Moreover, seed priming with these salts also reduced shoot  $\text{Na}^+$ , although the effect of KCl was more pronounced in reducing shoot  $\text{Na}^+$  contents (Iqbal and Ashraf 2007b). Seed priming with NaCl alleviated the adversities of salinity in pearl millet germination and later vegetative growth stage (Ashraf et al. 2002). Likewise, the rate of germination and synchronization was substantially improved by seed priming with NaCl in tomato under salinity stress (Cayuela et al. (1996)).

In a study on canola,  $\text{Na}^+$  contents of plants derived from primed seeds were lower, while  $\text{K}^+$  contents were relatively higher than those from non-primed seeds (Hassanpouraghdam et al. 2009). In maize, there was a substantial increase in  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  concentrations in germinating maize seeds primed with NaCl, KCl, or  $\text{CaCl}_2$ , respectively. Nonetheless, maximum  $\text{Cl}^-$  contents in germinating seeds were observed in seeds primed with  $\text{CaCl}_2$  followed by seeds primed with NaCl and KCl.

Resistance to salinity stress in wheat at germination was observed by osmopriming with different inorganic salts ( $\text{CaCl}_2$ , NaCl, and  $\text{CaSO}_4$ ),  $\text{CaCl}_2$  being the most effective priming agent (Iqbal et al. 2006a). Likewise, wheat seeds primed with  $\text{CaCl}_2$  germinated earlier than non-primed seeds.  $\text{CaCl}_2$  priming also improved total sugars and nonreducing sugars in wheat plants under salinity stress (Afzal et al. 2008). Ruan et al. (2002) reported improved seedling vigor index and seedling emergence and stand establishment in flooded soil by priming with  $\text{CaCl}_2$  and  $\text{CaCl}_2 + \text{NaCl}$ .

Several reports indicate that along with improved stand establishment, halopriming also enhanced subsequent growth, and ultimately final crop yield (Eleiwa 1989; Sallam 1999). For instance, halopriming improved growth of broad bean (*Vicia faba*; Sallam 1999), rice (Ruan et al. 2002), and maize (Farooq et al. 2008a–c); grain yield in wheat (Mehta et al. 1979) and soybean (Eleiwa 1989); and both growth and seed yield in *Pennisetum americanum* and sorghum (Kadiri and Hussaini 1999) and rice (Du and Tuong 2002) under stress conditions.

To summarize, halopriming is a cost-effective tool to improve crop stand establishment and subsequent growth and yield in stress-prone environments.

#### 41.2.1.2.3 Matrimpriming

In matrimpriming, seeds are mixed with moist solid matrix carriers having the ability to create matrix forces to hold water and facilitate its slow absorption by the seed (Khan 1992; Beckman et al. 1993; Taylor et al. 1998) such as granulated clay particles, sand, or vermiculite (Gray et al. 1990; Hardegree and Emmerich 1992a,b). After treatment, the seed is separated from the solid carrier, washed, and allowed to dry.

Matrimpriming improves crop performance under adverse field conditions. For example, sand priming improved cold tolerance in direct-seeded rice (Zhang et al. 2006); salinity resistance in maize was possibly due to increased soluble sugars and peroxidase (POD) and catalase (CAT) activity (Zhang et al. 2007b), and germination percentage of alfalfa under salinity stress (Hu et al. 2006); and speed of germination in three cultivars of maize. Matrimpriming contributed to shortened mean germination time and enhanced shoot height, seedling fresh and dry weights compared to unprimed control in maize (Zhang et al. 2007b), and germination and stand establishment of bitter melon (*Momordica charantia*) under chilling stress (Lin and Sung 2001).

In conclusion, though research reports are limited, matrimpriming has potential, and is a cheap alternative, for improving crop stress resistance.

#### 41.2.1.2.4 Priming with Hormones and Other Organic Sources

Soaking or treating seeds in optimal concentrations of plant growth regulators (PGRs) may improve germination, stand establishment, and growth and economic yield of crop plants under both normal and stress conditions (Darra et al. 1973; Hurly et al. 1991; Lee et al. 1998). Several growth regulators

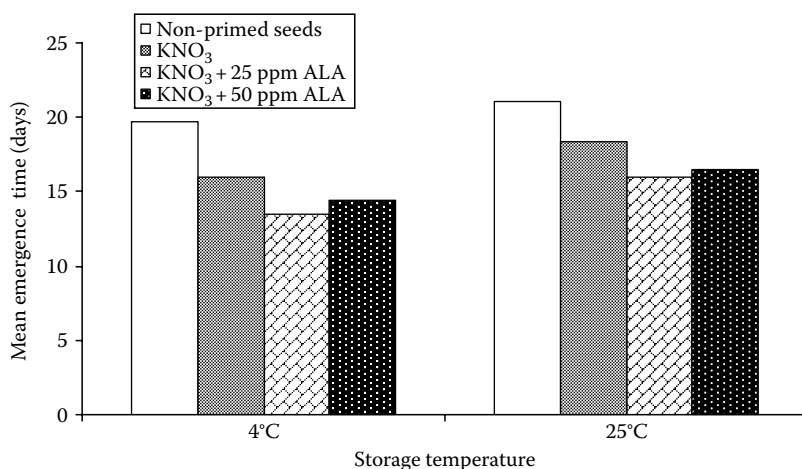


are commonly used for seed priming, including auxins (IAA, IBA, NAA), gibberellins (GAs), kinetin, abscisic acid (ABA), polyamines (PAs), brassinolide, salicylic acid (SA), triacontanol, and ascorbic acid (AA). Treating seeds with growth hormones improved crop germination under stressful conditions. Incorporating PGRs, PAs, and certain other organic sources in the priming solution and other pre-sowing treatments substantially improved resistance to abiotic stresses in many vegetable and field crops (Kim et al. 1993; Jeong et al. 1994; Iqbal and Ashraf 2005a,b; Farooq et al. 2008c, 2009ef).

In pigeon pea, seed priming with kinetin and AA was effective in mitigating the adverse effects of salt stress on germination (Jyotsna and Srivastava 1998). Likewise, wheat seed germination was substantially improved by soaking seed with IAA, NAA, or GA<sub>3</sub> under salt stress (Balki and Padole 1982). In another study, seed treatment with GA improved wheat germination under salinity stress (Parashar and Varma 1988). Similarly, wheat seed priming with a moderate concentration of kinetin (150 mg L<sup>-1</sup>) improved growth and grain yield possibly due to beneficial effects of kinetin on photosynthetic capacity and water-use efficiency under saline conditions (Iqbal and Ashraf 2005a). Gulnaz et al. (1999a) also reported similar findings with wheat seeds treated with IAA, IBA, or GA under salinity stress. Likewise, the adversities of salinity stress may be alleviated by seed priming with GA<sub>3</sub> in tomato (Kang et al. 1996) and okra (Vijayaraghavan 1999).

In field experiments, seed priming with tryptophan (Trp) improved wheat grain yield under salinity stress principally by reduction in Na<sup>+</sup> uptake and improved Ca<sup>2+</sup> partitioning in roots. There was increased free SA accumulation in wheat leaves under stress by seed priming with Trp and IAA (Iqbal and Ashraf 2007a). Kim et al. (2006) reported that although both GA and IAA improved salinity tolerance in rice, IAA was more effective.

Other plant growth hormones that have proven to be effective priming agents for improving growth or economic yield under stress conditions include 2,4-D, IAA, NAA, GA, AA, thiamin, and sodium salicylate in wheat (Al-Hakimi and Hamada 2001; Balki and Padole 1982; Gulnaz et al. 1999b; Parashar and Varma 1988); AA, thiamin, and pyridoxine in sunflower and maize (Ahmed-Hamad and Monsaly 1998); and 28-homobrassinolide in mungbean (Fariduddin et al. 2003). Several new compounds are also being tested for their potential use as priming agents. For example, incorporation of 5-aminolevulinic acid (25 and 50 ppm ALA) in the priming medium (KNO<sub>3</sub> solution) substantially improved the red pepper (*Capsicum annuum* cv. Sena) germination at low temperature stress (Korkmaz and Korkmaz 2009). They further added that primed seeds may be stored for a month (Figure 41.3).



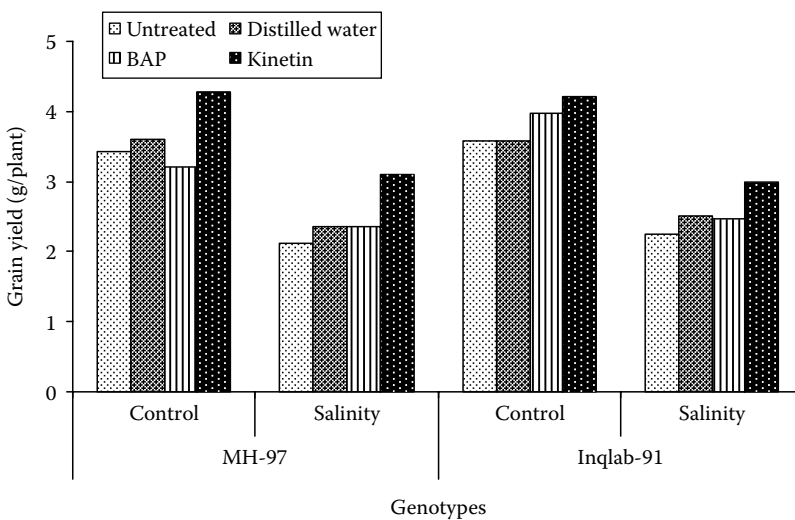
**FIGURE 41.3** Mean emergence time (MET) of “Sena” red pepper seeds emerged at 15°C following priming for 6 days at 25°C in 3% KNO<sub>3</sub> supplemented with 5-aminolevulinic acid (ALA) after 1 month storage at 4°C or 25°C. (Modified from Korkmaz, A. and Korkmaz, Y., *Sci. Hort.*, 119, 98, 2009.)

While there was improvement in pigeon pea by seed soaking in  $10 \text{ mg L}^{-1}$  kinetin (Verma and Srivastava 1998), in another study, Iqbal and Ashraf (2005a) evaluated the role of seed priming with cytokinins in improving wheat performance under salinity stress ( $15 \text{ dS m}^{-1}$ ). Seed priming with  $150 \text{ mg L}^{-1}$  kinetin was the most effective in improving net  $\text{CO}_2$  assimilation rate, water-use efficiency, growth, and grain yield. Seed priming with cytokinins also substantially improved germination and seedling growth under salinity stress. Salinity stress substantially reduced the grain yield, though seed priming with kinetin helped to lessen the reduction (Figure 41.4); nonetheless, there was a positive correlation of improved growth and increased grain yield with leaf IAA saline and normal conditions (Iqbal et al. 2006b). Akman (2009) found that priming with GA not only hastened seedling emergence but also overcame delaying effect of high concentrations of NaCl on germination.

Salicylate (SA), an endogenous growth regulator of phenolic nature, regulates several physiological processes (Raskin 1992) by interaction with other signaling pathways including the ones regulated by ethylene and jasmonic acid (Szalai et al. 2000; Ding and Wang 2003), and mineral uptake and membrane permeability, etc. (Barkosky and Einhelling 1993). SA has also been found to improve plant's resistance to abiotic stresses by activating glutathione reductase and guaiacol POD activities (Kang and Saltveit 2002). Korkmaz (2005) reported that priming pepper seeds in  $0.1 \text{ mM}$  of acetyl SA or  $3 \mu\text{M}$  methyl jasmonate incorporated into the  $\text{KNO}_3$  solution improved low temperature performance of sweet pepper seeds. Moreover, these seeds can be stored for 1 month at  $4^\circ\text{C}$ , which exhibited improved germination at  $15^\circ\text{C}$ .

In a recent study, we found that seed priming with SA improved chilling resistance in maize (Farooq et al. 2008c); while improved drought resistance in rice has also been reported by seed priming with SA and PAs (Farooq et al. 2009e,f). Hormonal priming of wheat seeds with AA and SA significantly improved emergence percentage, root and shoot lengths, and fresh and dry weights of wheat seedlings under both saline and nonsaline conditions (Afzal et al. 2005). Wheat seeds primed with  $50 \text{ ppm}$  SA and AA not only improved final germination counts but also reduced germination time under saline conditions. Seedlings grown from seeds treated with  $50 \text{ ppm}$  SA had higher root lengths and fresh and dry shoot weights than non-primed seeds (Afzal et al. 2006b).

Likewise, seed priming with SA significantly improved the seedling growth in wheat under water stress (Singh and Ushu 2003). It is thought that the protective and growth-promoting effect of SA was due to increased mitotic activity within the apical meristem of seedling roots



**FIGURE 41.4** Grain yield of two cultivars of hexaploid wheat at  $2.18 \text{ dS m}^{-1}$  (control) or  $15 \text{ dS m}^{-1}$  NaCl when the seeds were primed in cytokinin or benzylaminopurine (BAP) solutions ( $200 \text{ mg L}^{-1}$ ). (Modified from Iqbal, M. and Ashraf, M., *J. Integr. Plant Biol.*, 47, 1315, 2005a.)

(Sakhabutdinova et al. 2003). Khodary (2004) also reported that SA increased shoot and root fresh and dry weights of stressed maize plants.

PAs have a profound influence on the crop performance under variety of environmental conditions (Watson and Malmberg 1998). As PAs are cationic in nature, these may make association with membrane phospholipids, help in stabilizing the bilayer structure, and reduce injuries to membrane upon exposure to environmental stresses (Basra et al. 1994). Thus, polyamine accumulation may help to protect the plants from adversities of environmental stresses (Bouchereau et al. 1999). Seed priming with PAs (putrescine, spermidine, and spermine) reduced leaf free-ABA levels in salt-sensitive wheat cv. MH-97 than the control plants under salinity stress; nonetheless, there was a substantial increase in leaf ABA contents by seed treatment with putrescine (Put) in the same cultivar under stress conditions (Iqbal and Ashraf 2006). In a laboratory experiment, there was substantial increase in germination and seedling vigor at low temperature in rice by soaking seeds in putrescine, spermidine, spermine, betaine, and praline (Naidu and Williams 2004). While examining the effect of PAs as priming agents, Iqbal and Ashraf (2005b) reported that PAs (viz., spermine, spermidine, putrescine) may be used as a priming agent effectively to minimize the adversities of salinity stress in various wheat cultivars; nonetheless, the effect of PAs was cultivar specific.

Brassinosteroids (BRs) can also be used to induce stress tolerance. For example, rice seeds treated with BRs reversed the inhibitory effect of salinity on germination and seedling growth (Anuradha and Rao 2001). In alfalfa, seed priming with brassinolide significantly increased the activities of antioxidant enzymes, superoxide dismutase (SOD), POD, and CAT in Victoria and Victor seedlings. In addition, during seedling growth, primed seeds manifested significantly reduced MDA accumulation. Sand priming also enhanced the activities of CAT, POD, SOD, and soluble sugar content and reduced MDA accumulation under salt stress (Hu et al. 2006). Zhang et al. (2007a) reported that alfalfa seed priming with brassinolide increased shoot fresh and dry weights, root dry weight, root length, and root vigor.

Seed priming with AA substantially increased the ascorbate leaf contents, thereby reducing the ROS levels in various wheat cultivars under salinity stress (Afzal et al. 2006a). This supports the hypothesis that increased ROS is the primary cause of seedling mortality under these conditions. Al-Hakimi and Hamada (2001) found that seed priming with AA exerted favorable effects on seedling growth and water relations in wheat counteracting the inhibitory effects of salinity stress, seed priming with AA alleviated the adversities of salinity stress principally with improved leaf soluble proteins, thereby protecting the cellular membranes (Jeng and Sung 1994). This improved leaf protein contents also improved the allometry and growth under saline conditions (Afzal et al. 2006a).

In summary, presoaking seed treatments with PGRs and other organic substances of similar nature may improve crop resistance to abiotic stresses if used at optimum concentrations. Since PGRs are costly, field application is unlikely unless commercial formulations at affordable prices are developed.

#### 41.2.1.2.5 *Priming with Low Molecular Weight Osmolytes and Stress Signaling Molecules*

Osmolytes help maintain cytoplasmic turgor pressure during water stress and stabilize the structure and functions of certain macromolecules, and ultimately promote plant growth under stressful conditions (Mickelbart et al. 2003). Seed treatment of these solutes may be advantageous as they improve stress tolerance of plants (Agboma et al. 1997). For example, there was significant improvement in drought resistance in rice by seed priming with glycinebetain (GB). Seed priming helped in maintaining the tissue water status and improving antioxidant systems, thereby improving the cellular membrane integrity, and enabled the plants to grow well even in limited water supply (Farooq et al. 2008e,f). Likewise, priming of maize seeds with GB substantially improved stand establishment and growth under chilling stress (Farooq et al. 2008b). Soaking rice seeds in various combinations of IAA and GB was also effective in inducing low temperature tolerance in rice. Furthermore, the

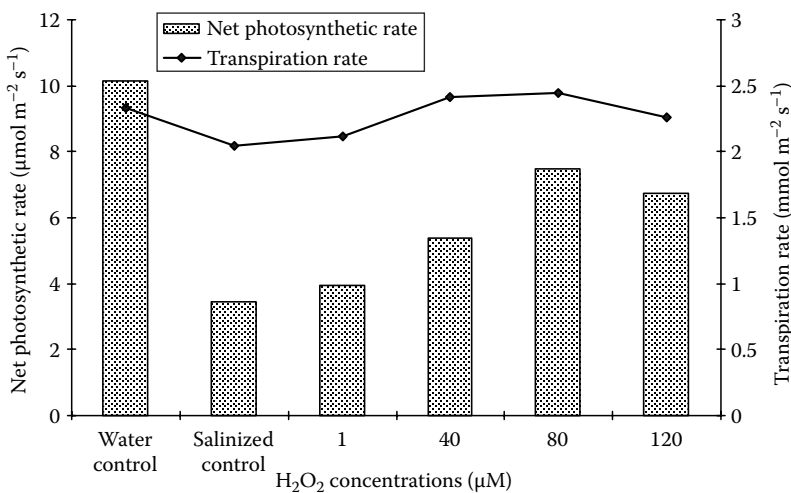
combined application of IAA and GB was more effective in rice seed performance than individual effects (Chen et al. 2005).

Posmyk and Janas (2007) primed mungbean seeds in proline and water. Both priming treatments improved seed germination and seedling growth at 5°C; however, seedlings primed with proline had the best growth. In barley, seed treatment with GB indicated higher shoot dry weight, net photosynthesis, leaf water potential, and reduced relative membrane permeability compared to untreated plants under high temperature. GB might have been absorbed by seeds and translocation to seedlings to enhance their capacity to maintain higher water content and seedling vigor with increased photosynthesis, reduced membrane permeability, and leakage of important ions under heat stress (Wahid and Shabbir 2005).

Exogenous use of stress-signaling molecules such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ethylene oxide, or nitric oxide as seed treatments or foliar sprays has potential to improve stress tolerance (Zhu 2002; Taiz and Zeiger 2006; Wahid et al. 2007b, 2008). For example, wheat seed treated with 1–120 mM  $\text{H}_2\text{O}_2$  improved the stand establishment. Seed treatment with  $\text{H}_2\text{O}_2$  helped the seedlings to sustain the carbon-assimilation and dry matter accumulation (Figure 41.5; Wahid et al. 2007b). Furthermore, there was a substantial increase in heat-stable (stress) proteins with apparent molecular weight of 32 and 52 kDa and higher  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$  uptake in  $\text{H}_2\text{O}_2$ -treated seedlings (Wahid et al. 2007b). Seedlings expressed two heat-stable (stress) proteins when treated with  $\text{H}_2\text{O}_2$ . Sasaki et al. (2005) also reported growth promotion by  $\text{H}_2\text{O}_2$  treatment under low temperature in a greenhouse.

Wheat seed priming with sodium nitroprusside (SNP) significantly alleviated the reduced  $\beta$ -amylase activity but had little effect on  $\alpha$ -amylase activity of wheat seeds under salt stress. SNP slightly increased  $\alpha$ -amylase isoenzymes (especially isoenzyme 3) and significantly increased  $\beta$ -amylase isoenzymes (especially isoenzyme d, e, f and g). SNP pretreatment decreased  $\text{Na}^+$  content and increased  $\text{K}^+$  content, resulting in a marked increase in the  $\text{K}^+:\text{Na}^+$  ratio of wheat seedlings under salinity. These data suggested that NO is involved in promoting wheat seed germination under salt stress by increasing  $\beta$ -amylase activity (Duan et al. 2007). Similarly, seed priming with SNP (NO donor) substantially improved seedling growth, water relations, and general metabolism under drought stress in rice (Farooq et al. 2009g).

In summary, seed treatment with low molecular weight osmolytes and stress-signaling molecules may effectively improve crop resistance against abiotic stresses; hence, their application on a commercial scale may be beneficial.



**FIGURE 41.5** Changes in gas exchange of wheat leaves under increased  $\text{H}_2\text{O}_2$  concentrations with or without salinity. In water control neither the salinity was imposed nor seeds treated with  $\text{H}_2\text{O}_2$  were applied; while in salinized control under salinity stress (150 mM)  $\text{H}_2\text{O}_2$  was not applied. (Modified from Wahid, A. et al., *J. Plant Physiol.*, 164, 283, 2007b)

### 41.2.2 THERMAL TREATMENTS

Thermal treatments refer to seed incubation at low or high temperatures for certain periods of time to improve germination and seedling emergence under adverse environmental conditions. Traditionally, dry heat treatment of seeds has been used for two purposes: first, to control internal and external seed-borne pathogens including virus, bacteria, fungi, and nematodes (Nakagawa and Yamaguchi 1989; Fourest et al. 1990); and second, to break seed dormancy (Zhang 1990; Dadlani and Seshu 1990). In general, high temperatures in dry heat treatments reduce seed viability and seedling vigor, but are optimal for breaking dormancy to promote rice seed germination and seedling emergence (Lee et al. 2002).

Pre-sowing chilling treatment of seeds in some species is a common practice in agriculture either to protect seeds from precocious germination in unsuitable environments or to improve germination (Bewley and Black 1994). For instance, Sharma and Kumar (1999) reported that pre-sowing chilling treatments improved salinity tolerance in Indian mustard (*Brassica juncea*) possibly due to increased  $K^+$  and  $Ca^{2+}$ , and reduced  $Na^+$  uptake. In another study, chilling treatment of pearl millet (*Pennisetum glaucum*) seed for 2 days at 5°C increased final germination percentage, but not germination rate, under saline conditions (Ashraf et al. 2003). Similar mitigation of adverse effects of salt stress by chilling seed has been observed in Indian grass (*Sorghastrum nutans*) (Watkinson and Pill 1998) and parsnip (*Pastinaca sativa*) (Finch-Savage and Cox 1982).

In summary, although limited information is available regarding the potential of thermal treatments in improving crop resistance against abiotic stresses, such treatments may improve seedling establishment and crop performance under stress conditions, and their potential needs to be explored.

### 41.2.3 SEED COATING AND PELLETING

In seed coating, required materials are applied so that they may influence the seed or soil at the seed–soil interface. Seed coating generally refers to the application of finely ground solids or liquids containing dissolved or suspended solids to form a more or less continuous layer covering the natural seed coat; it includes pelleting and many other seed treatments (Scott 1989).

Much of the literature related to seed coatings reports *ad hoc* testing of various chemicals, coating materials, additives, etc., applied to seeds in ways that are often ill-defined. To date, there has been little effort to view seed coating as a branch of science that needs many basic principles to be understood before progress can be made. In this regard, Heydecker and Coolbear (1977) were concerned that pelleting technology was not progressing because manufacturers kept their materials and processes a closely guarded secret.

Seed pelleting with lime is being used in several grain and pasture legumes, e.g., soybean, clover, and alfalfa, to improve the stand establishment in the soils with low pH (Pijnenborg and Lie 1990). Nonetheless, the main objective of these treatments is to improve the effective nodulation by protecting rhizobia on inoculated seed in low pH soils (Norris 1973; Cordero and Blair 1978). Rice seed is pelleted with calcium peroxide to increase oxygen availability in submerged paddy conditions (Halmer 1988, 1994).

Murata et al. (2008) reported that seed pelleting with sulfate, chloride, nitrate, and carbonate of calcium or Calcimax reduced seedling mortality in acidic soils. Pelleting the groundnut seed with Ca improved plant growth. However, calcium carbonate was most effective at reducing seedling mortality. In another study, Zhang et al. (2007c) investigated the mechanism involved in rice cold resistance by seed-coating agents and concluded that rice seedlings treated with cold-tolerant seed-coating agents under chilling stress maintained much higher root vigor, POD, CAT, and SOD activities, and chlorophyll contents, had lower MDA contents and electrolyte leakage, accumulated more soluble sugars and free proline, and lower plant injury rates when compared with the control.

In conclusion, seed coating and pelleting treatments may have potential on a field-scale, with the help of private enterprises to formulate and market seed for a particular environment.

### 41.3 ON-FARM PRACTICAL APPLICATIONS OF INVIGORATION TECHNIQUES

The findings of any research endeavor remain unproductive until adapted by the end user. It is evident from recent research efforts that by soaking seeds in water followed by surface drying before sowing (on-farm priming), many crop species germinate faster, emerge earlier, and have more vigorous seedling growth, resulting in higher yielding crops (Harris et al. 1999, 2000; Musa et al. 1999). On-farm seed priming is a simple, low-cost, low-risk method for promoting rapid seedling establishment, vigorous and faster seedling growth.

Harris et al. (2002) reported that in upland rainfed areas, seedling emergence, stand establishment, allometry, phenology, and final yield may be improved substantially if primed rice seeds are used. Several farmers now use seed invigoration techniques to boost crop performance under adverse soil conditions. Of these, on-farm seed priming is the most popular in farming communities (personal observation).

### 41.4 CONCLUSION

Seed invigoration tools have the potential to improve emergence and stand establishment under a range of field conditions. Of particular interest are hydropriming, osmopriming, halopriming, hormonal priming, and using highly soluble and low molecular weight chemicals. These techniques may enhance crop performance under saline, submerged, and drought-prone conditions, and on marginal land. Variation exists within crops and varieties/genotypes/hybrids in their response to various priming treatments, which will enable researchers to identify useful accessions for further work such as the following:

- More precise invigoration techniques using a range of salts, PGRs, jasmonates, and osmolytes at varying concentrations and for different durations.
- Optimal water potential, temperature range, and requirements for oxygenation.
- Commercial fertilizers as priming and seed coating agents.
- Performance of invigorated seeds under a wide range of field conditions.
- Thermal treatments with alternate cycles of low and high temperatures.
- Storage potential of primed seeds—prolonged storage of primed and hardened seeds may be critical for technology transfer and marketing of primed seeds.

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# 42 Plant Stress Physiology: Physiological and Biochemical Strategies Allowing Plants/Crops to Thrive under Ionic Stress

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42.1 INTRODUCTION

Abiotic stresses, such as drought and salinity, are serious threats to agriculture and the natural status of the environment. They are recurring features of nearly all the world’s climatic regions since various critical environmental threats with global implications have linkages to water crises (Gleick, 1994, 1998, 2000). These threats are collaterally catalyzed by global warming and population growth.

The latest scientific data confirm that the earth’s climate is rapidly changing. Global temperatures have increased by about 1°C over the course of the last century, and will likely rise even more rapidly in the coming decades (Intergovernmental Panel on Climate Change, 2007). Scientists predict that temperatures could rise by another 3°C–9°C by the end of the century with far-reaching effects. Increased drought and salinization of arable land are expected to have devastating global effects (Wang et al., 2003a). The current amount of annual loss of arable area could double by the end of the century because of global warming (Evans, 2005). Simultaneously, rapid population growth increasingly generates pressure on existing cultivated land and other resources (Ericson et al., 1999). Population migration to those arid and semiarid areas increase the problems of water shortage and worsens the situation of land degradation in the destination, and in turn causes severe problems of poverty, social instability, and population health threats (Figure 42.1, Moench, 2002). Water scarcity and desertification could critically undermine efforts for sustainable development, introducing new threats to human health, ecosystems, and national economies of several countries. Therefore, solutions to these problems are desperately needed, such as the improvement of salinity tolerance of crops. Two different experimental approaches to increase crop salt tolerance are in use: (1) growing plants in saline conditions and comparing their performance to a control group grown under optimal conditions and (2) applying salt stress after plants have been grown under optimal conditions for a while. These approaches investigate how an individual plant species adapts to a saline environment and what its stress response is like, respectively. This second approach is widely used, but its

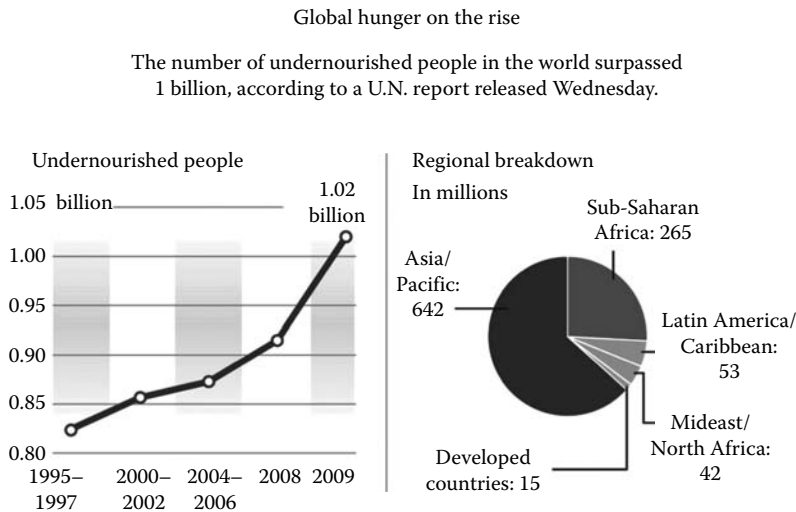
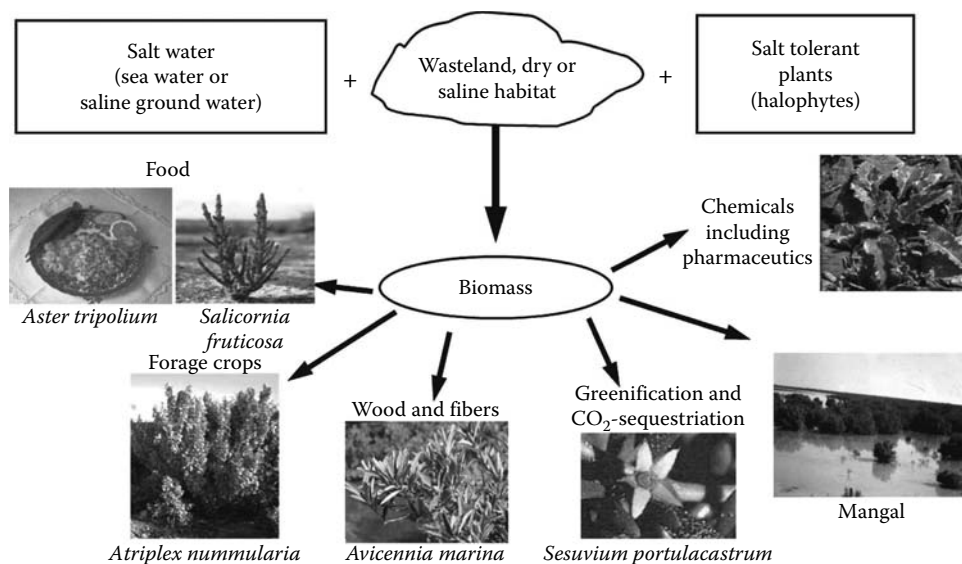


FIGURE 42.1 Global hunger on the rise. (From Food and Agriculture Organization of the United Nations.)





**FIGURE 42.2** Sustainable use of cash-crop halophytes.

meaning has been questioned a lot as well: As a rule, salt stress does not occur suddenly in the field. Moreover, the combination of this approach with current micro-array technique provided too many hits to be handled in subsequent breeding approaches. Apparently most of the genes would respond to any type of stress. They are activated in response to disturbed homeostasis rather than specific response to salt stress (Kawasaki et al., 2001).

Some eco-physiologists have analyzed performance of plants differing in their salt tolerance and have introduced the term “halophytes” for plants showing high salt tolerance. Some of these plants can tolerate seawater irrigation (Lieth and Menzel, 1999); most of these plants are obligate halophytes, i.e., they show optimal growth only in the presence of some salt (100–200 mM, these are concentrations already toxic for many other plants). It is a matter of ongoing research to breed halophytes for cropping purpose (Figure 42.2, sustainable use of so-called cash-crop halophytes). Another approach under discussion is, whether genes from halophytes can be transferred to regular crops to make them more salt tolerant (Flowers et al., 1997; Glenn et al., 1998).

A more recent idea is to take advantage of the genetic potential crops still have (Glenn et al., 1998). This idea requires to first identify accessions of a crop species showing enhanced salt tolerance. In a second step, genes (enzymes or metabolic pathways) have to be identified that result in stress tolerance. Finally, early indicators of salt tolerance have to be identified allowing successful breeding (Ashraf, 1999; Koyro et al., 2009). Moreover, understanding the physiological and biochemical basis of stress tolerance can help to identify proper culture conditions. This may allow extending the cultivation area of an individual species to regions having suboptimal environmental conditions.

## 42.2 PHYSIOLOGICAL ASPECTS

### 42.2.1 HALOPHYTES: PLANTS ABLE TO THRIVE ON SALINE SUBSTRATES

Despite the importance of salinity in shaping the composition of coastal plant communities, our understanding of how different species respond physiologically to variable salinities is limited (Touchette et al., 2009). Halophytes are plants that are able to complete their life cycle in a substrate rich in NaCl (Schimper, 1891). Approximately 2600 halophytes are known worldwide, and they constitute 1% of the world’s flora (Lieth and Menzel, 1999; Flowers and Comer, 2008).



**FIGURE 42.3** Salt excretion of the mangrove *Avicennia marina* L.

In contrast to most glycophytic crops, they still have not lost resistance mechanisms to salt stress conditions. These may take the form of salt avoidance or tolerance (Yeo, 1983, 1998; Touchette et al., 2009). They are classified as euhalophytes (true halophytes), pseudohalophytes (salt avoiders), or crinohalophytes (salt excreters), according to their apparent adaptation to environmental salinity (Figure 42.3, Ungar, 1991).

Some plants (the Mojave Desert Star, for instance) avoid the effects of high salt by fancy tricks such as by completing the reproductive life cycle during rainy seasons (facultative halophytes). Nevertheless, the bandwidth of resistance mechanisms is larger in obligatory halophytes or xerohalophytes (drought-tolerant halophytes). These are plants tolerating salinities higher than 0.5% NaCl (Koyro and Lieth, 1998). For obligate halophytes, saline substrates even offer advantages for the competition with glycophytes.

Although the terms glycophytes and halophytes insemminate the impression that there are general qualitative differences in adaptation, in reality things are more complex. There is a fluent passage between these two groups or may be a just stereotype thinking owed to the human wish of differentiation. Additionally, almost all of the salt adaptive mechanisms underlie the physiological and ecological complexity as well as structural changes.

Because of that, information about the salinity tolerance of glycophytes and halophytes needs partially careful checking. Furthermore, a prerequisite for the sustainable utilization of plants (such as suitable halophytes) on saline sites is the precise knowledge about the various mechanisms enabling a plant to grow at (their natural) saline habitats (Marcum, 1999; Warne et al. 1999; Weber and D'Antonio, 1999; Winter et al., 1999). This chapter reviews the eco-physiological mechanisms.

#### **42.2.2 EXPERIMENTAL PROOF FOR THE SUITABILITY OF PLANTS**

As freshwater resources will become limited in near future (Lieth, 1999), it is necessary to develop sustainable biological production systems, which can tolerate higher water salinity. A precondition is the identification and/or development of salinity-tolerant crops. First of all, halophytes have to be studied in their natural habitat and a determination of all environmental demands has to be completed. On the basis of this information, the selection of potentially useful plants should begin (Lieth, 1999). The first step of this identification list contains the characterization and classification of the soil and climate, under which potentially useful halophytes grow. Only artificial conditions in sea water irrigation systems in a growth cabinet under photoperiodic conditions offer the possibility to study potentially useful halophytes under reproducible experimental growth and substrate conditions. The supply of different degrees of sea water salinity

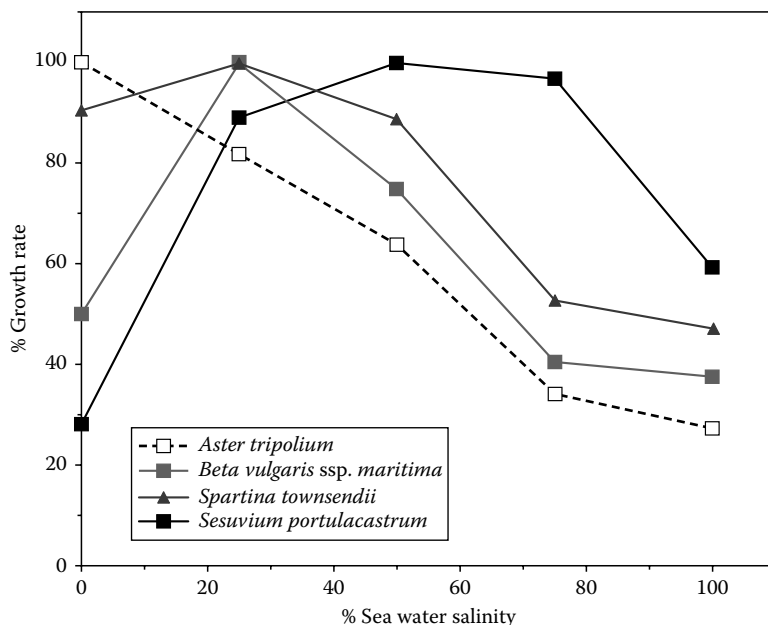


**FIGURE 42.4** Quick check system of halophytes. Gravel/hydroponic quick check system (QCS) with automatic drip irrigation under photoperiodic conditions in a growth cabinet (plant species: *Beta vulgaris* ssp. *maritima*). Controls are visible in the foreground, the sea water salinization treatment in the background.

[0%, 25%, 50%, 75%, 100% (and if necessary higher) sea water salinity] to the roots in separate systems under otherwise identical or/and close to natural conditions gives the necessary preconditions for a comparative study in a quick check system (QCS) for potential cash crop halophytes (Figure 42.4; Koyro and Huchzermeyer, 1999a). It is well known that salinity tolerance depends on the stage of development and period of time over which the plants have grown in saline conditions (Munns, 2002). Plants were exposed to salinity in the juvenile state of development and were studied until achieving the steady state of adult plants. Variable applicable QCS seems to be valuable for the selection of useful plants and it suggests itself as a first step for the controlled establishment of “cash crop halophytes” because it provides detailed information about three major goals as there are the threshold of salinity tolerance at idealized growth conditions, how to uncover the individual mechanisms for salt tolerance and about the potential of utilization for the preselected halophytic species.

### 42.2.3 THRESHOLD OF SALINITY TOLERANCE

In correspondence with the definition for the threshold of salinity tolerance according to Kinzel (1982), the growth reaction and the gas exchange are used during the screening of halophytes as objective parameters for the description of the actual condition of a plant (Ashraf and O’Leary, 1996). Reliable information is now available about studies with several halophytic species from different families such as *Aster tripolium*, *Inula crithmoides*, *Plantago* cf. *coronopus*, *Laguncularia racemosa*, *Limoniastrum articulatum*, *Beta vulgaris* ssp. *maritima*, *Atriplex nummularia*, *Atriplex leucoclada*, *Atriplex halimus*, *Chenopodium quinoa*, *Batis maritima*, *Puccinellia maritima*, *Spartina townsendii*, and *Sesuvium portulacastrum* (Pasternak, 1990; Koyro and Huchzermeyer, 1997, 1999a; Koyro et al., 1999; Lieth and Menzel, 1999; Koyro, 2000; Koyro and Huchzermeyer, 2004a; Geissler et al., 2009a). The substrate concentration leading to a growth depression of 50% (refer to freshweight, in comparison to plants without salinity) is easy to calculate with the QCS (by extrapolation of the data) and it leads to a precise specification of a comparative value for the threshold of salinity tolerance. Dramatic differences are found between halophytic plant species. The threshold of salinity tolerance amounts to  $300 \text{ mol m}^{-3}$  NaCl in *Aster tripolium*,  $375 \text{ mol m}^{-3}$  in *Beta vulgaris* ssp. *maritima*,  $500 \text{ mol m}^{-3}$  in *Spartina townsendii*, and  $750 \text{ mol m}^{-3}$  in *Sesuvium portulacastrum* (Figure 42.5). These results prove that it is essential to quantify differences in salinity tolerance between halophytic species as one basis for assessment of their potential of utilization.



**FIGURE 42.5** Threshold of salinity tolerance. Dramatic differences are found between halophytic plant species. The threshold of salinity tolerance amounts to  $300 \text{ mol m}^{-3}$  NaCl in *Aster tripolium*,  $375 \text{ mol m}^{-3}$  in *Beta vulgaris ssp. maritima*,  $500 \text{ mol m}^{-3}$  in *Spartina townsendii*, and  $750 \text{ mol m}^{-3}$  in *Sesuvium portulacastrum*.

#### 42.2.4 MAJOR CONSTRAINTS OF PLANT GROWTH ON SALINE SUBSTRATES

The salinity tolerance of halophytic plants is in most cases multigenic; it comprises a wide range of morphological, physiological, and biochemical mechanisms on whole plant, tissue, and cellular/molecular levels (Wang et al., 2003a,b; Ashraf and Harris, 2004). Only rarely a single parameter is of major importance for the ability to survive at high NaCl salinity. A comprehensive study with the analysis of at least a combination of several parameters is a necessity to get a survey about the mechanisms which in the end leads to the salinity tolerance of individual species. These mechanisms are connected to the four major constraints of plant growth on saline substrates: (1) water deficit, (2) restriction of  $\text{CO}_2$  uptake, (3) ion toxicity, and (4) nutrient imbalance.

Plants growing in saline habitats face the problem of a low water potential in the soil solution and high concentrations of potentially toxic ions such as chloride and sodium. Salt tolerance involves physiological and biochemical adaptations for maintaining protoplasmic viability while cells compartmentalize electrolytes. Salt avoidance involves structural and physiological adaptations to minimize salt concentrations of the cells or physiological exclusion by root membranes. In principle, salt tolerance can be achieved by salt exclusion or salt inclusion. Salt exclusion minimizes ion toxicity but accelerates water deficit and indirectly diminishes  $\text{CO}_2$  uptake. Salt absorption facilitates osmotic adjustment but can lead to toxicity and nutritional imbalance. The following physiological mechanisms to avoid salt injury (and to protect the symplast) are known as major plant responses to high NaCl salinity (Marschner, 1995; Mengel and Kirkby, 2001; Munns, 2002; Koyro and Huchzermeyer, 2004a):

1. Regulation of the water potential, decrease of the osmotic and matrix potential, enhanced synthesis of organic solutes.
2. Optimization of the gas exchange ( $\text{H}_2\text{O}$  and  $\text{CO}_2$ ), high water use efficiency (of photosynthesis ( $\text{H}_2\text{O}$  loss per net  $\text{CO}_2$  uptake), ion radical scavenging, or/and switch to CAM-type of photosynthesis.

3. Maintenance of ion homeostasis especially in the cytoplasm of vital organs
  - a. Selective uptake or exclusion (e.g., salt glands).
  - b. Selective ion transport in the shoot, in storage organs, to the growing parts and to the flowering parts of the plants, re-translocation in the phloem.
  - c. Compartmentation of Na and Cl in the vacuole or cell wall.
4. High storage capacity for NaCl in the entirety of all vacuoles of a plant organ, generally in old and drying parts (e.g., in leaves supposed to be dropped later) or in special structures such as hairs. The dilution of a high NaCl content can be reached in parallel by an increase in tissue water content (and a decrease of the surface area, succulence).
5. Tolerance of high NaCl concentrations in the symplast.
6. Conversion of whole plant metabolism to high NaCl concentrations (synthesis of NaCl-tolerant enzymes, osmolyte biosynthesis, ion-protecting agents such as proline and glycinebetaine).
7. Restricted diffusion of NaCl in the (root-) apoplast.
8. Anatomical modification.

These mechanisms will be reviewed in more detail in the following sections.

#### 42.2.5 REGULATION OF THE WATER RELATIONS AND OPTIMIZATION OF THE GAS EXCHANGE

According to Munns, plants show a two-phase growth response to salinity (Munns, 1993, 2002; Munns et al., 2002). The first phase of growth reduction is essentially a water stress or osmotic phase and presumably regulated by hormonal signals coming from the roots.

Terrestrial plants in saline habitats are often surrounded by low water potentials in the soil solution and atmosphere. For water to flow through the soil–plant–atmosphere continuum, a gradient of decreasing water potentials ( $\Psi$ ) must be established. The  $\Psi$  of pure water is defined as 0 MPa; increasing salinity or concentrations of other solutes will decrease  $\Psi$ . Thus, any sharp rise in salinity could effectively hold water osmotically away from plants that lack any physiological or morphological modifications (Larcher, 2003). To limit restrictions on water uptake, plants must generate increasingly lower  $\Psi$  to allow continued water flux into belowground structures (Touchette et al., 2009).

It is also important to prevent water loss by transpiration from being higher than the influx rate. This is only possible if the water potential remains lower in the plant than in the soil. However, data demonstrated clearly that leaf water potential of halophytes does not correlate alone as a single factor with salinity tolerance. Plant species with different levels of salt tolerance such as *Aster tripolium*, *Beta vulgaris* ssp. *maritima*, *Spartina townsendii*, and *Sesuvium portulacastrum*, have a sufficient adjustment mechanism even at high salinity treatment. Clearly, tolerance in the form of osmotic adjustment plays an important role in halophytes residing in saline environments (Flowers and Colmer, 2008). However, in addition were the osmotic potentials of all four halophytes (and many others) up to sea water salinity level sufficiently low to explain the full turgescence of the leaves (results not shown). Except for differences in concentration and type of osmotica used in plant tissues (ions are more prevalent in halophytes), physiological responses in plants to salt stress were remarkably similar to those employed during drought (Touchette et al., 2009). For plants with limited water availability, physiological adjustments often involve avoidance and tolerance, with most plants using some combination of the two (Yue et al., 2006; Romanello et al., 2008).

Assuming there is no interruption of the water supply, water can flow passively from the root to the shoot and there seems to be no reason for growth reduction by water deficit for any of the studied species. However, by regulating the extent of apoplastic barriers and their chemical composition (long-distance response coordination), plants can effectively regulate the uptake or loss of water and solutes (by structures such as barriers in the hypo- or exodermis). This appears to be an additional or compensatory strategy of plants to acquire water and solutes (Hose et al., 2001) and at the extremes of growth under conditions of drought and high salinity make the exodermis an

absolute barrier for water and ions in the strict sense (Azaizeh and Steudle, 1991; North and Nobel, 1991; Nublat et al., 2001).

Thus, the rate of supply of water to the shoot can be restricted due to the coupling between the flows of water and solutes (Na and Cl) even if the leaf water potential is low. Therefore, the balance between water flow (sum of water accumulation and transpiration) and the decrease in the amounts of nutrients or unfavorable nutrient ratios (e.g.,  $\text{Na}^+/\text{K}^+$ ) are important factors for impaired leaf elongation (Lynch et al., 1988; Munns et al., 1989; Neves-Piestun and Bernstein, 2001; Cramer, 2003) and plant growth.

In any case, plant water loss has to be minimized at low soil water potentials, since biomass production depends mainly on the ability to keep a high net photosynthesis by low water loss rates. In this field of tension, biomass production of a plant has always to be seen in connection with the  $\text{CO}_2/\text{H}_2\text{O}$ -gas exchange, which can be estimated by the water use efficiency (WUE) of photosynthesis. A critical point for the plant is reached if the  $\text{CO}_2$  fixation (apparent photosynthesis) falls below the  $\text{CO}_2$  production (compensation point). Therefore, one crucial aspect of the screening procedure is the study of growth reduction, water consumption, and net photosynthesis especially at the threshold of salinity tolerance (Geissler et al., 2009a).

Several halophytic plants such as *Aster tripolium*, *Beta vulgaris* ssp. *maritima*, *Chenopodium quinoa*, or *Spartina townsendii* reveal a combination of low (but positive) net photosynthesis, minimum transpiration, high stomatal resistance, and minimum internal  $\text{CO}_2$  concentration at their threshold salinity tolerance (Koyro, 2000; Koyro and Huchzermeyer, 2004a). However, there is a big bandwidth among halophytes, especially for succulent halophytes such as *Sesuvium portulacastrum* or *Avicennia marina*, which have alternatives if the water balance is still positive (water uptake minus water loss) and not the limiting factor for photosynthesis. In case of *S. portulacastrum*, net photosynthesis and WUE increase but stomatal resistance decreases. These results show that it is quite important to describe the regulation of gas exchange at high salinity in strong reliance with other parameters (such as water relations). Water deficit is one major constraint at high salinity and can lead to a restriction of  $\text{CO}_2$  uptake and to the development of radical oxygen species. The balance between water loss and  $\text{CO}_2$  uptake helps to find weak spot in the mechanism of adjustment (of photosynthesis) to high salinity (Badawi et al., 2004).

#### 42.2.6 MAINTENANCE OF ION HOMEOSTASIS: AVOIDANCE OF ION EXCESS AND ION IMBALANCE

There is a second phase of growth response to salinity which takes time to develop, and results from internal injury (Munns, 1993, 2002; Mengel and Kirkby, 2001; Boström et al., 2003). It is due to ion-specific effects, i.e., salts accumulating in transpiring leaves to excessive levels.

Ion toxicity and nutrient imbalance are two major constraints of growth at saline habitats and therefore of special importance for the salt tolerance of halophytes. For some ions (such as  $\text{Na}^+$  and  $\text{K}^+$ ), either their excess or deficiency has been found to be toxic to freshwater and marine organisms. Adverse effects can occur in plants on saline substrates when common ions exceed a certain concentration, when the normal composition (ratio) of ions is not correct, or in some cases, when ion concentrations are too low.

Data of additional scientific studies have shown that halophytes exhibit very different ways of adjustment to high NaCl salinity. There are several known examples, where salt-tolerant plants differ from salt-sensitive relatives in having a lower rate of  $\text{Na}^+$  and  $\text{Cl}^-$  transport to leaves (Munns, 2002). However, some halophytes even need an excess of salts for maximum growth and for attaining low solute potentials (Flowers et al., 1977; Greenway and Munns, 1980). This is much less energy consuming than synthesizing organic substances (Yeo, 1983; Chaves et al., 2009). Nevertheless, high substrate salinities can lead to toxic effects of salt even inside these includers (Munns, 2005). The cause of injury is probably the salt load exceeding the ability of cells to compartmentalize salts in the vacuole. Salts would then build up rapidly in the cytoplasm and inhibit enzyme activity. Alternatively, they might build up in the cell walls and dehydrate the cell.

High  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations can be avoided by filtering out most of the salt. Plants with this ability are often called excluders in literature (in contrast to includers). The use of these terms is highly debated because they are mainly used to provide a clearer understanding although they are again just stereotypes with a limited validity. However, ion-excluding halophytes have to synthesize osmotically active solutes within the plant to meet turgor pressure demands (Mengel and Kirkby, 2001). This adaptive feature can be of importance even in species that have salt glands or bladders.

For the sake of completeness, it has to be said that it is quite important to distinguish between both ions to uncover the individual mechanisms for salt tolerance. The mechanisms of salt exclusion are discussed in literature mainly as if a common reaction of both ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) is leading to salt injury. This is not always the case. Some halophytes such as *Laguncularia racemosa* (with salt glands) is a typical  $\text{Na}$  excluder but with high  $\text{Cl}^-$  accumulation in the leaves (Koyro et al., 1997), and *Beta vulgaris* ssp. *maritima* is a typical  $\text{Cl}^-$  excluder with high  $\text{Na}^+$  accumulation in the leaves (Kinzel, 1982; Koyro and Huchzermeyer, 1999a).

Halophytes are able to distinguish precisely between the metabolic effects of both ions  $\text{Cl}^-$  and  $\text{Na}^+$ : Some halophytes such as *Scirpus americanus*, *Avicennia marina* (salt excreter with salt glands), or *Rhizophora mangle* are able to exclude  $\text{Na}^+$  and  $\text{Cl}^-$  (see literature in Kinzel, 1982) from the leaves, *Laguncularia racemosa* (salt excreter with salt glands) diminish the  $\text{Na}^+$  but not the  $\text{Cl}^-$  concentration in the leaves (Koyro et al., 1997), *Beta vulgaris* ssp. *maritima*, *Suaeda brevifolia*, *Suaeda vera*, *Limoneastrum monoptalum*, *Allenrolfea occidentalis*, or *Spartina townsendii* accumulate much higher  $\text{Na}^+$  than  $\text{Cl}^-$  in the leaves (see literature in Kinzel, 1982; Koyro and Huchzermeyer, 1999a) and *Salicornia rubra*, *Salicornia utahensis*, *Suaeda occidentalis*, *Atriplex vesicaria*, *Atriplex nummularia*, *Atriplex papula*, *Atriplex rosea*, or *Inula crithmoides* accumulate  $\text{Na}^+$  and  $\text{Cl}^-$  in the leaves in a range above the saline environment (salt includers). Typical halophytic adaptation includes in this case leaf succulence in order to dilute toxic ion concentrations (Kinzel, 1982; Mengel and Kirkby, 2001) or they perform salt excretion (e.g., with bladder hairs such as all listed *Atriplex* species).

If plants accumulate  $\text{Na}^+$  and  $\text{Cl}^-$  in concentrations not sufficient to balance the external water-potential, a lack of solutes may result in adverse effects on water balance, so that water deficiency rather than salt toxicity may be the growth-limiting factor (Greenway and Munns, 1980; Mengel and Kirkby, 2001). To achieve a low water potential and/or a charge balance, the solute potential in these species is decreased by the synthesis of organic solutes such as sugar alcohol (e.g., mannitol in leaves of *Laguncularia racemosa*), soluble carbohydrates (e.g., sucrose in taproots of *Beta maritima* ssp. *maritima*), organic acids (incl. amino acids), or by reducing the matric potential (e.g., with soluble proteins in leaves of *Beta vulgaris* ssp. *maritima*). However, the synthesis of organic solutes is energy demanding and the formation of these solutes decreases the energy status of the plant (Yeo, 1983; Chaves et al., 2009). Thus, for plant survival, growth depression is a necessary compromise in  $\text{Na}^+$  and/or  $\text{Cl}^-$  excluding species and not a sign of toxicity or nutrient imbalance.

#### 42.2.6.1 Protection of the Cytoplasm

It can be distinguished between two salt-specific effects. One is leading to a reduction of the entry of salt into the plant and the other one regulates a low concentration of salt in the cytoplasm. Both effects contribute to root and leaf cytosolic  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in the order of 10–30 mM (Wyn Jones and Gorham, 2002; Tester and Davenport, 2003; Koyro and Huchzermeyer, 2004b).

The disturbance of metabolism by  $\text{Na}^+$  or  $\text{Cl}^-$  has to be avoided if plants have to grow on saline habitats. Therefore, the protection of the responsible enzymes by maintaining low cytosolic sodium concentrations is of major importance (Borsani et al., 2003). Indeed, leaves being fed by the transpiration stream receive large quantities of sodium, which must be regulated. Plant cells respond to salt stress by increasing sodium efflux at the plasma membrane and sodium accumulation in the vacuole. For such a reason, the proteins, and ultimately genes, involved in these processes can be considered as salt-tolerance determinants. The cloning experiments of  $\text{Na}^+/\text{H}^+$  antiporter have demonstrated the role of intracellular sodium (Ohta, 2002) compartmentalization in plant salt

resistance. Such compartmentation of sodium and chloride in leaf vacuoles can only be attained with an active transport into the vacuole and low permeability of the tonoplast to these ions.

The transport of ions across the plasma membrane and tonoplast requires energy, which is provided by plasma membrane and vacuolar ATPase, respectively (Koyro and Huchzermeyer, 1997; Leigh, 1997).  $\text{Na}^+/\text{H}^+$  antiporters, for instance, take advantage of a proton gradient formed by these pumps. Salt stress was shown to increase  $\text{Na}^+/\text{H}^+$  activity in glycophytes and halophytes (Apse and Blumwald, 2002). The activation of such antiporters is likely to be operating to reduce sodium toxicity in salt-tolerant plants under saline conditions.

#### 42.2.6.2 Selective Ion Accumulation

High salt concentrations can also cause intracellular ionic imbalances (such as of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{SO}_4^{2-}$ ) (Mengel and Kirkby, 2001). The capacity of plants to maintain  $\text{K}^+$  homeostasis and low  $\text{Na}^+$  concentrations in the cytoplasm appears to be one important determinant of plant salt tolerance (Yeo, 1998; Läuchli, 1999). A possibility to find such limiting factors is the study of the relations inside single cells such as the compartmentation between cytoplasm and vacuole, the distribution of elements in different cell types or along a diffusion zone in a root apoplast and ultrastructural changes.

*Beta vulgaris* ssp. *maritima* and *Spartina townsendii* (salt excreter) both have high  $\text{Na}^+$  accumulation in the leaves. Both species seem to react similar to salinity with changes in leaf water potential, gas exchange, and nutrients. However, this number of congruence does not allow to conclude analogical intracellular relations. The comparison of their intracellular ionic balance will be used to demonstrate the necessity of special physiological investigations.

In contrast to water stress effects that occur in the meristematic region of younger leaves, the effects of ion toxicity predominantly arise in mature leaves (Mengel and Kirkby, 2001). This is because  $\text{Na}^+$  and  $\text{Cl}^-$  are stored mainly in the shoot of halophytes such as *Beta vulgaris* ssp. *maritima* and *Spartina townsendii* leading to a growth reduction of the aboveground parts much higher than of the root (Koyro, 2000; Koyro and Huchzermeyer, 2004a). These changes can be interpreted as signs of a critical load. Therefore, to distinguish between the individual mechanisms of salinity tolerance, further investigations of the intracellular ionic balance were performed first of all at epidermal leaf cells (the end of the transpiration stream) of both these species.

However, the intracellular composition of the leaf epidermal cytoplasm and vacuoles of controls of *Beta vulgaris* ssp. *maritima* and *Spartina townsendii* show some more congruities of both species. The epidermal vacuoles of controls of both species contain most of the elements (with the exception of  $\text{PO}_4^{3-}$ ) in higher concentrations as the cytoplasm indicating the overall picture of a vacuolar buffer. The leaf vacuoles in its entirety can be described as a voluminous potassium pool with high storage capacity for sodium and chloride. This pool is needed in case of high  $\text{NaCl}$  salinity for the maintenance of the  $\text{K}$ -homeostasis in the cytoplasm. The dominant elements in the cytoplasm were  $\text{PO}_4^{3-}$  and  $\text{K}^+$ . The  $\text{K}^+$ -concentrations were in the epidermal cytoplasm of control plants in an ideal range for enzymatic reactions (Wyn Jones et al., 1979; Wyn Jones and Pollard, 1983; Koyro and Stelzer, 1988).

It is obvious that seawater salinity leads to a decrease of  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^+$  in the epidermal vacuoles of both species. The remaining  $\text{K}^+$ ,  $\text{SO}_4^{2-}$ , and  $\text{Mg}^{2+}$  concentrations were only in *Spartina* two digit and especially for  $\text{K}^+$  much higher as in *Beta*. The vacuolar buffer of the latter one seems to be exhausted.

$\text{NaCl}$  salinity led to a significant decrease of the  $\text{K}^+$  and  $\text{PO}_4^{3-}$  concentrations especially in the cytoplasm of *Beta* and to a breakdown of the homeostasis. This result points at a deficiency for both elements in the cytoplasm. Additionally, the concentrations of sodium and chlorine were at high  $\text{NaCl}$  salinity below  $5 \text{ mol m}^{-3}$  in the cytoplasm of the epidermal cytoplasm and the gradients between cytoplasm and vacuole were higher in comparison with the results of *Spartina*. In summary, these results support the hypothesis that the sea beet does not sustain ion toxicity but ion deficiency! It is hypothesized that such low  $\text{K}^+$  levels in the cytoplasm can lead to a reduction of protein



synthesis, which is of utmost importance in the process of leaf expansion (Mengel and Kirkby, 2001). One possible consequence is the supply of sufficient fertilizers (especially  $K^+$  and  $PO_4^{3-}$ ) at high NaCl salinity to reduce the symptoms of  $K^+$  and  $PO_4^{3-}$ -deficiency in *Beta*.

The salt-induced reductions of the cytoplasmic  $K^+$  and  $PO_4^{3-}$  concentrations were much less pronounced in *Spartina* as in *Beta*. The results of *Spartina* point at a working system to keep ionic homeostasis. However, there was one important exception: The sodium concentration increased significantly in the epidermal cytoplasm. Sodium could (to some extent) substitute potassium in its cytoplasmic functions or it could be the first sign of an intoxication.

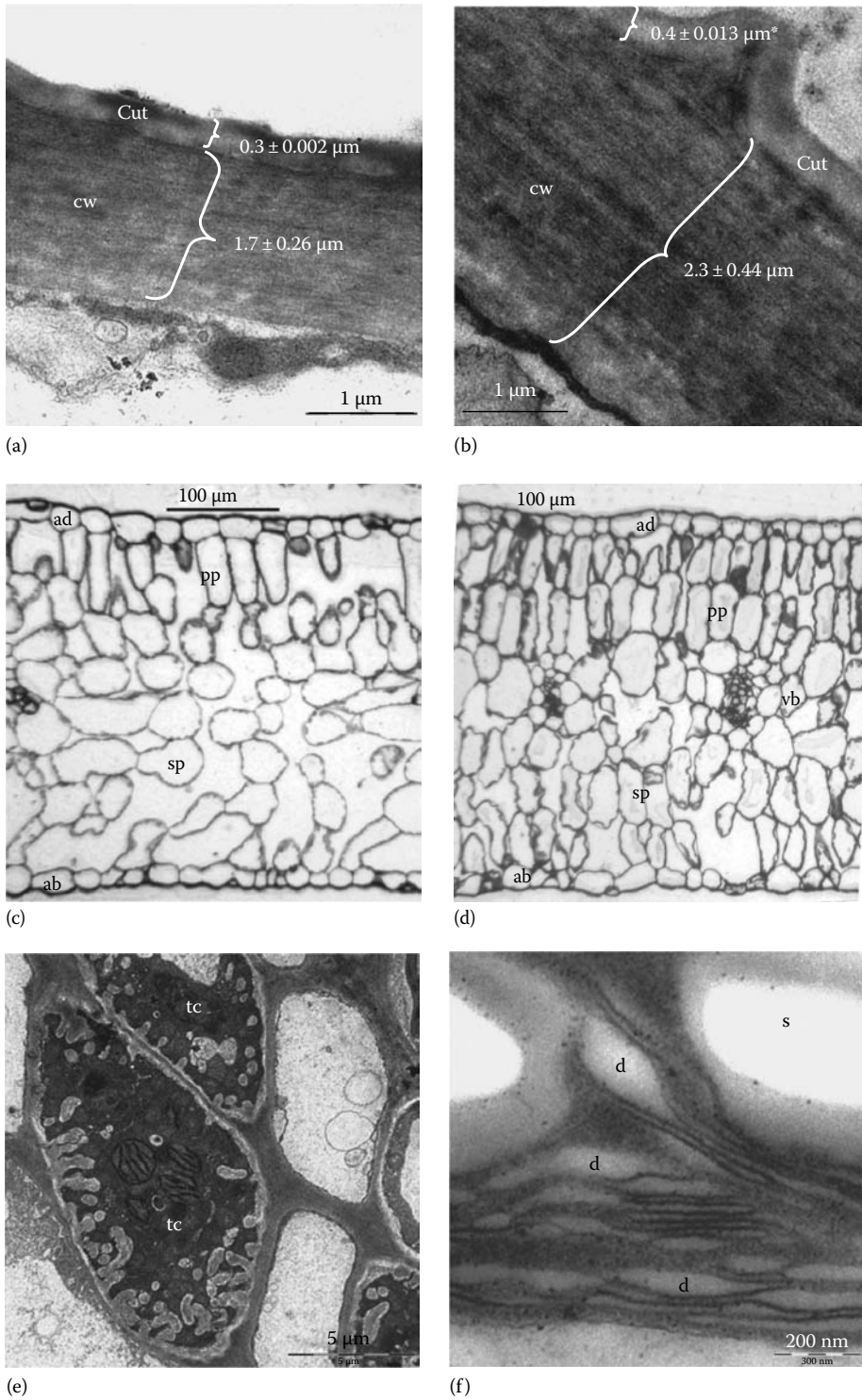
The results and interpretations are in agreement with the hypothesis that plant growth is affected by ion imbalance and toxicity and probably leads to the long-term growth differences between the salt-tolerant and salt-sensitive species.

However, *Beta* and *Spartina* are also two excellent examples of how important it can be to validate intracellular ionic imbalances ( $K^+$ ,  $Ca^{2+}$ , and  $Mg^{2+}$ ) at high salt concentrations to uncover the individual mechanisms for salt tolerance and to understand the threshold levels of individual species.

#### 42.2.7 MORPHOLOGICAL ADAPTATION

In many cases, various mechanisms and special morphological structures are advantageous for halophytes since they help regulate tissue salt concentrations or minimize water loss and oxidative stress (Figure 42.6, Marschner, 1995; Breckle, 2002; Koyro, 2002; Geissler et al., 2009b).

- Succulence and/or a high LMA (leaf mass-to-area ratio) is demonstrated in many genera of plants that inhabit saline environments, such as *Suaeda maritima*, *Sesuvium portulacastrum*, or even *Aster tripolium*. By depositing ions of salts in large vacuoles, the toxicity is partitioned from the cytoplasm and organelles of the cells.
- A laterally extensive, shallow root system enables the plant to optimize water and nutrient uptake.
- Many halophytes exhibit a rather rapid turnover of their leaves, e.g., in rosette species such as *Aster tripolium* salts are stored in older leaves and removed from the plant when these leaves are shed.
- Excretive halophytes have glandular cells capable of excreting excess salts from plant organs (salt excreter). A simple system with two-celled trichomes that have evolved as collecting chambers for salts, e.g., in *Spartina townsendii*, *Glaux maritima*, *Triglochin maritimum*, and a complex type of salt glands is known e.g., in several mangroves such as *Avicennia marina* or *Limonium vulgare*.
- Not only glands but also bladder hairs can remove salts from salt-sensitive metabolic sites. Some halophytes like *Atriplex* or *Chenopodium* have vesiculated trichomes on the leaf surface which release the salt back into the environment when they are ruptured.
- Halophytes often exhibit reflecting surfaces (by wax such as *Spartina* ssp. or trichomes such as *Avicennia marina*) preventing ultraviolet light from reaching the leaf tissues and therefore minimizing the development of reactive molecules (reactive oxygen species, ROS, as well as nitrogen radicals).
- Curled leaves, fine hairs, waxy cuticle, high stomate density, a small stomate size, sunken stomata, elevations on the surface such as bulliform cells support the buildup of an unstirred layer at the leaf surface and reduce transpiration and thus import of salt. For example, in *Aster tripolium* salinity leads to a significant increase in cuticle and cell wall thickness of the epidermal leaf cells.
- A decrease in intercellular spaces is often observed with increasing salinity in order to reduce transpiration, such as in *Aster tripolium* or *Beta vulgaris* ssp. *maritima*.
- An increased number of vesicles under saline conditions and transfer cells in the vascular bundles facilitate selective ion transport processes, such as in *Aster tripolium*.



**FIGURE 42.6** Structural features of *Aster tripolium*. (a) Cell wall and cuticle of the upper leaf epidermis, control; (b) cell wall and cuticle of the upper leaf epidermis, 375 NaCl; (c) cross section of leaf, control; (d) cross section of leaf, 375 NaCl; (e) transfer cell in leaf vascular bundle (phloem); (f) dilations of thylakoid membranes, 375 NaCl.

Although halophytes have developed morphological structures (and physiological mechanisms) that help regulating tissue salt concentrations or to minimize water loss and oxidative stress, they may show structural symptoms of disorders under high salinity levels (Yamada et al. 2009). Dilations of cell membranes such as the thylakoid membranes in the chloroplasts are often found not only in salt-stressed glycophytes, but also in halophytes (Fidalgo et al., 2004; Paramanova et al., 2004; Geissler et al., 2009b). Such kind of damage has been discussed by many authors as a consequence of oxidative stress (Mitsuya et al., 2003; Oksanen et al., 2005), but it may also be the result of ion toxicity or imbalance (Keiper et al., 1998; Yamane et al., 2003; Geissler et al., 2009a,b).

#### 42.2.8 SUSTAINABLE USE OF HALOPHYTES

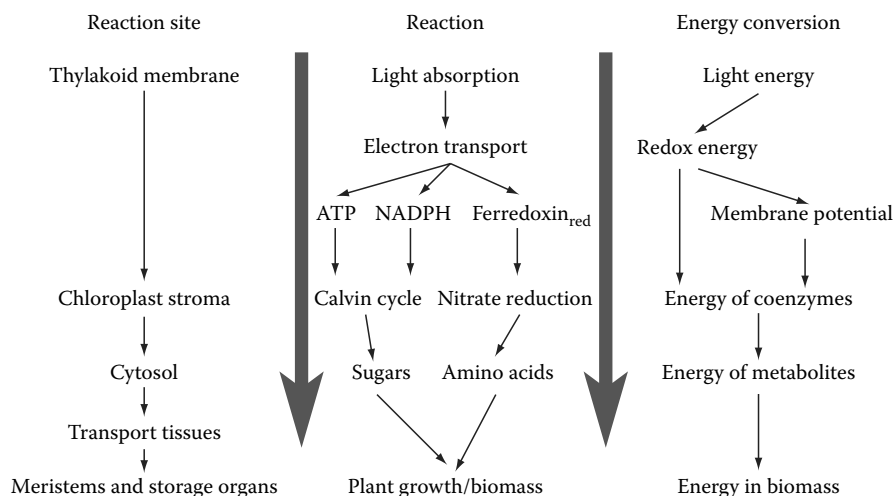
Physiological studies using the sea water irrigation system have the potential to provide highly valuable means of detecting individual mechanisms of species against NaCl stress, and may also provide opportunities for the comparison and screening of different varieties for their adaptation to salinity (QCS for cash crop halophytes). After the selection of halophytic species suited for a particular climate and for a particular utilization, green house experiments at the local substrates (and climatic conditions) to select and propagate promising sites (Isla et al., 1997) have to be started. This must be followed by studies with lysimeters on field site to study the water consumption and ion movements. Last but not least, a design for a sustainable production system in plantations at coastal areas or at inland sites (for example, for economical use) has to be developed.

### 42.3 BIOCHEMICAL ASPECTS

#### 42.3.1 INHIBITION OF PRIMARY REACTIONS OF PHOTOSYNTHESIS

Limitation of plant growth by environmental factors is a matter of general concern especially with respect to crop production and food and feed supply. Photosynthesis is dominating plant growth and production of biomass. Therefore, the sequence of reactions leading to the phenomenon named photosynthesis is in the focus of interest when breeding for high crop yield. In order to allow detailed analysis of salt effects, several individual steps have to be distinguished (Figure 42.7).

At first, there are primary reactions of photosynthesis, namely, absorption of light energy and (1) its conversion to redox energy, conserved in the coenzyme NADPH, and (2) energy of chemical



**FIGURE 42.7** Photosynthetic energy flow. Reaction sequence of photosynthesis can be described in terms of reaction sites (left column), sequence of reactions (center), conversion of energy forms (right column).

bounds, conserved in the coenzyme ATP. On the second level, we find reactions of the Calvin cycle, nitrate and sulfate reduction as well as sugar, lipid, and amino acid metabolism. Typical reactions we have to discuss on the third level are trans membrane and inter tissues transport of metabolites. The fourth level of photosynthesis relates to physiological aspects of gas exchange, turgor homeostasis, etc.; these aspects already have been discussed above.

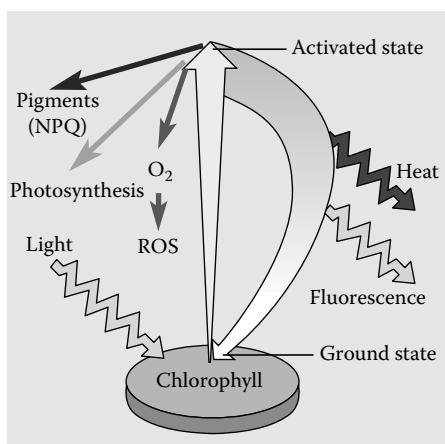
In the literature, we find several publications on salt stress effects on photosynthesis (see, for example, Chaudhuri and Choudhuri, 1997; Rajesh et al., 1998; Soussi et al., 1998; AliDinar et al., 1999; Kurban et al., 1999; Kao et al., 2001; Romeroaranda et al., 2001). Mostly, it is stated that salt stress response of plants is a multifactorial trait. This statement is correct, for sure. But it does not provide sufficient help for breeders and farmers requesting advice what traits to look at and how to treat crops in the field properly.

In many papers analyzing salt stress effects on photosynthesis, more precise answers could have been achieved, if authors would have described more precisely what they really have been measuring. The general impression is that most papers deal on salt effects on physiological aspects of biomass production rather than partial reactions of photosynthesis as defined above. As there are cross reactions as well as cell signaling involved in regulation of photosynthetic metabolic pathways, there are no strict correlations between biomass production and individual gene activities. As a consequence, predicted correlations, though they had been observed in laboratory experiments, could not be shown in subsequent field experiments. Here, we try a more differentiated approach and review analysis of salt stress effects at different levels of photosynthesis.

It will become very obvious that no individual laboratory is able on its own to approach the problem of salt effects on plant growth in an appropriate way. Working on plant salt stress physiology requires cooperation among teams of different expertise.

#### 42.3.1.1 Photosynthetic Conversion of Energy

In plants active in photosynthesis, energy of light quanta is absorbed by chlorophyll. Just like all other pigments, activated chlorophyll can return from its activated state to the stable, nonactivated state by emitting heat. Other than most pigments, chlorophyll after absorption energy of a red light quantum is sufficiently stable in its activated state to allow transfer of energy to acceptor molecules of biological relevance (Strasser et al., 2004; [Figure 42.8](#)). In principle, there are the two options of biological relevance in addition to a third one we can use for monitoring of plant performance: (1) resonance energy transfer to activate other pigments and (2) transfer of an electron to an acceptor. The electron acceptor can be a component of the photosynthetic electron transport chain or any



**FIGURE 42.8** Competition for absorbed energy of light quanta. Photosynthetic electron transport is competing with “futile reactions” for energy of light-activated chlorophyll.

other molecule having a less negative potential as compared to activated chlorophyll. This second option requires a “refill” of electrons, and it is well documented that in chloroplasts the water splitting system is functioning that way in order to have chlorophyll recycled to its ground state again (Strasser et al., 2004). (3) Another option, competing for energy with the already mentioned ones, is the emission of fluorescence energy (Schreiber, 1997; Schreiber et al., 2002; Strasser et al., 2004). As will be discussed later, any inhibition of an individual pathway will enhance the possibility of the other pathways to occur.

In thylakoid membranes, photosystems I and II can be distinguished from other chlorophyll-containing protein complexes, the light-harvesting complexes. Only the special pairs of chlorophyll<sub>a</sub> located in the active centers of the two photosystems are involved in electron transport. Energy transfer among other pigments occurs via resonance energy transfer. In this section, we focus on electron transfer reactions.

As known from the literature, half lifetime of activated state of chlorophyll is very short and depends on the environment of the pigment (Rees et al., 1990; Laible et al., 1994; Ma et al., 2009). It is obvious that efficiency of photosynthesis depends on the probability that activated chlorophyll will transfer electrons to an acceptor of the photosynthetic electron transport chain rather than “wasting” energy by using one of the other pathways mentioned above (Ruban et al., 1993; Horton et al., 1996; Horton, 2000; Allen and Forsberg, 2001). In order to meet this requirement, reaction partners are arranged in ideal neighborhood within protein complexes located in the thylakoid membranes. Moreover, it has been demonstrated that this structure undergoes permanent adjustment to match the requirement of chloroplast metabolism and to adapt to changes in the environment, i.e., changes of light quality and intensity, for instance (Allen and Bennett, 1981; Allen, 1992; Allen and Forsberg, 2001).

By means of photosynthetic electron transport, energy of absorbed light quanta is converted to redox energy stored in the coenzyme NADPH and proton motive force (Mitchell, 1967) stored in a proton gradient across the thylakoid membranes. This proton gradient is the driving force for ATP synthesis catalyzed by the chloroplast F-type ATPase, called the CF<sub>0</sub>CF<sub>1</sub>-complex or the chloroplast coupling factor (Strotmann et al., 1976; Huchzermeyer and Strotmann, 1977; Boyer, 2000). The number of coenzyme molecules (NADP<sup>+</sup>/NADPH and ADP/ATP) is limited, and there is no exchange of coenzymes among cell compartments. Therefore, it is required for efficiency of this machinery that acceptor forms of coenzymes, NADP<sup>+</sup> and ADP, respectively, permanently are recycled by subsequent metabolic pathways. Otherwise, turnover of energy by the electron transport would be inhibited, and this inhibition finally would lead to an inhibition of electron release from activated chlorophyll, enhancing the probability of alternative routes mentioned above (Figure 42.8).

Photosynthetic CO<sub>2</sub> assimilation is the major consumer recycling both coenzymes in the reaction sequence of the Calvin cycle. Under physiological conditions, as a rule of thumb, in chloroplasts of nonwoody plants two-thirds of the electrons from the noncyclic electron transport pathway finally will be consumed by CO<sub>2</sub> fixation while one-third will be used for nitrate reduction (see top part of Figure 42.10) (Schmidt and Jäger, 1992). But it has to be mentioned here that a significant portion of the absorbed light energy will be “wasted” in futile reaction sequences rather than be used for biomass synthesis.

#### 42.3.1.2 Salt Effects on Photosynthetic Energy Conversion

In order to understand individual reactions of photosynthesis, and finally get an overview of the principles how they interact, thylakoid membranes and protein complexes have been isolated and analyzed with respect to their structure and function. During preparation and subsequent tests of enzyme activities high salt concentrations (50 mM NaCl as a rule) have been applied without any inhibitory effect on individual enzyme activities (Strotmann et al., 1976). Such high salt concentrations are not found inside chloroplasts, neither under physiological conditions nor under salt stress. It therefore can be concluded that primary reactions of photosynthesis are not directly inhibited under salt stress (Richter et al., 2000; Huchzermeyer et al., 2004; Huchzermeyer and

Koyro, 2005; Koyro and Huchzermeyer, 2005). But this conclusion disagrees with apparent inhibition of photosynthesis observed in whole plant experiments (Lawlor and Fock, 1978; Lawlor, 2002). Therefore, it has to be analyzed in more detail, at what extent the observed inhibition may be attributed to salt-dependent changes in thylakoid structure (Hesse et al., 1976) or other effects described in the physiology and transport section of this chapter. One first approach to answer this question could be a detailed analysis of the kinetics of fluorescence light emission subsequent to chlorophyll activation by light pulses. Application of the pulsed amplitude modulation technique (PAM) allowed a detailed analysis of the network of primary reactions in photosynthesis (Strasser et al., 2004). It became quite obvious that salt stress does not directly inhibit primary reactions of photosynthesis but inhibits product export and fine-tuning of primary reactions by interfering with optimal arrangement of proteins and membranes (Koyro and Huchzermeyer, 1999b; Huchzermeyer and Heins, 2000; Huchzermeyer, 2000).

Accordingly, it was observed that maximal photochemical efficiency, indicated by high Fv/Fm values of chlorophyll fluorescence, remain high under tolerable salt stress, while growth rate of turf grass, for instance, was reduced under the same salinity level (Lee et al., 2004).

It will be discussed in subsequent paragraphs how salt can inhibit export of products of photosynthesis and why proper function of the phosphate translocator, located in the inner envelope membrane, is essential for photophosphorylation (see Section 42.3.4). At this stage, we can state that inhibition of product export feeds back to primary reactions and finally will inhibit photosynthetic electron transport. One option to release energy from its activated state is blocked and chlorophyll will increase activity of fluorescence light emission, heat production, energy transfer to other pigments, and ROS production. This latter option will be discussed in the following paragraph (see Section 42.3.2).

Though leguminosae like peas and beans are of high importance, we will not comment on special aspects of photosynthesis linked to symbiosis. From current literature it becomes obvious that in these crops nitrogen fixation in root nodules is more sensitive to salt stress as compared to CO<sub>2</sub> fixation. In chick pea, for instance, it was found that this apparent high sensitivity is due to interference of incoming salt with malate transport to the bacteroides (Soussi et al., 1998). Discrimination of primary and secondary salt stress effects in general is complicated, and primary targets within the metabolic network of hosts and symbionts are not easy to identify.

#### 42.3.1.3 Salt Effects on Chloroplast Structure and Metabolite Transfer

Chloroplasts easily can be sedimented by centrifugation because of their very high protein content. Süß and coworkers argued that inside the chloroplast stroma, proteins tend to interact and form aggregates. Indeed, they were able to isolate such “super complexes” and found out that they contain enzymes belonging to individual metabolic pathways (Süß et al., 1993). Such an arrangement helps to increase substrate turnover, because diffusion distances among enzymes of a pathway are close to zero. For the same reason, formation of such aggregates prevents occurrence of side reactions, because intermediates are not available for other enzymes. As will be discussed in Section 42.3.3.1, salt on one hand can inhibit turnover of substrates by destroying enzyme aggregates. On the other hand, intermediates of sugar metabolism, for instance, can become available for other enzymes and alternative products (compatible solutes, for instance) can be formed. Though salt effects on metabolite patterns have been analyzed in several papers, no data linking these findings to the occurrence of protein aggregates are available to date.

The occurrence of thylakoid grana stacks has attracted a lot of interest for years (Huchzermeyer et al., 1986; Lam and Malkin, 1989; Malkin and Braun, 1993; Romanowska and Albertson, 1994). It was calculated that about 70% of all PSII complexes are located within stacked regions of the thylakoid membranes, while most of the PSI complexes are found in un-stacked part of the thylakoid membranes (Schmidt and Malkin, 1993; Romanowska and Albertsson, 1994). From this, it was concluded that 70% of the PSII complexes must be in an inactive state, because the distance between PSII and PSI would be too far to allow sufficient turnover rates of photosynthetic electron transport



(Malkin and Braun, 1993). Moreover, it was found that grana are unstable, permanently folding and unfolding structures. Apparently  $CF_0CF_1$ -complexes are initiating formation of membrane loops (Boekema et al., 1988) and grana are formed by interaction of membrane proteins (Staehelin, 1975). As active PSII has an extremely short half lifetime in the range of 20–30 min (Kuhn and Böger, 1990; Trebst and Soll-Bracht, 1996; Keren et al., 1997; Jansen et al., 2001), the function of this permanent modification of membrane structure may be, to initiate interaction among membrane proteins and to support building of aggregates of protein complexes interacting in photosynthetic electron transport chain. Such preferred neighborhood of membrane proteins has been found, indeed (Laszlo et al., 1984; Huchzermeyer and Willms, 1985). There are several publications indicating that such neighborhoods of protein complexes are controlling efficiency of energy conversion in primary reactions of photosynthesis (see papers on localized protons, for instance, Laszlo et al., 1984; Löhr and Huchzermeyer, 1985; Löhr et al., 1985; Allnutt et al., 1989). Moreover, they might be controlling photo-inhibition as well (Lu and Zhang, 1999; Lu et al., 2002).

From the above description, it is obvious that formation of grana stacks is essential for optimal functioning and permanent repair of photosynthetic electron transport rate. It has been found that grana become destabilized if the ratio of monovalent and divalent cations is impaired (Hesse et al., 1976). Thus, it may be argued that salt stress does not directly inhibit individual primary reactions of photosynthesis but will impair adjustment to environmental conditions and destabilize structures allowing optimal energy use. Until now salt-tolerant and salt-sensitive plant species have not been analyzed for differences in this respect. Analysis of the situation becomes more complicated by recent findings: Apparently repair of light and ROS stressed photosystem II can be impaired on translation level of the D1 protein via interference with photorespiration (Takahashi et al., 2007). This way, inhibition of metabolite transfer between chloroplasts, peroxisomes, and mitochondria would feed back on photoinhibition. Data on salt stress effects on photosystem I of higher plants currently are not available. But investigation of primary events of photosynthesis in green algae indicate that assembly of PSI subunit structure is impaired by incoming salt (Allakhverdiev et al., 2000) (more recent publication in preparation).

## 42.3.2 SALT-INDUCED PRODUCTION OF POTENTIAL TOXIC INTERMEDIATES OF PHOTOSYNTHESIS

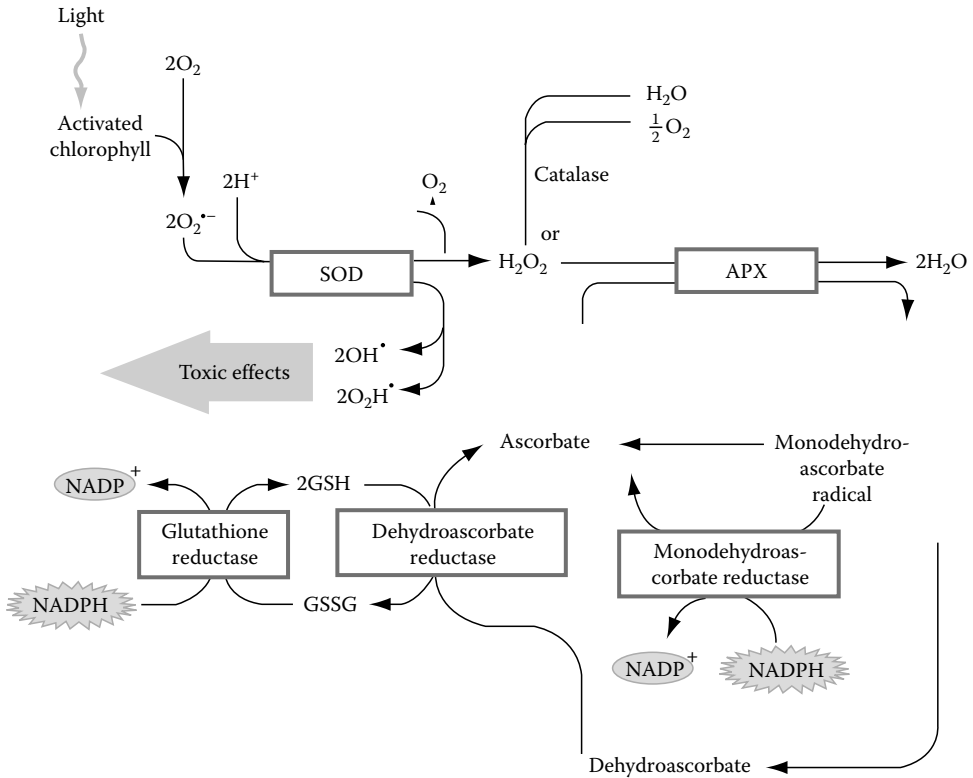
### 42.3.2.1 ROS

#### 42.3.2.1.1 *The Physiological Situation*

As described above, any reduction of electron transport rate, especially under high light conditions, will increase the risk of ROS production (see [Figure 42.8](#)). Concentration of these reactive compounds will build up in the light and eventually will reach concentrations toxic for cells active in photosynthesis.

The redox potential of activated chlorophyll is more negative than the one of oxygen. Therefore electron transfer from activated chlorophyll can occur unless energy is abstracted from activated chlorophyll faster than electron transfer to oxygen can occur. There are further options of ROS production as several intermediates of photosynthetic electron transport are radicals. On the other hand, ROS can spontaneously convert or can be turned over under the control of enzymes ([Figure 42.9](#)). Accordingly, in the literature the occurrence of various forms of ROS is described (Halliwell and Gutteridge, 1986; Elstner, 1987). Cytotoxicity may be attributed to oxidative damage of membrane lipids (Fridovich, 1986; Wise and Naylor, 1987) as well as oxidation of proteins and nucleic acids (Fridovich, 1986; Imlay and Linn, 1988).

In the field, salt stress results in severe damage especially in situations when its inhibitory effects occur in presence of high light intensity. Then PSII activity will result in high oxygen concentrations, especially if stomata are closed under stress. Concomitantly, chlorophyll will remain in its active state for a prolonged period of time, as electron transport rate is reduced by inhibited off flow of products. Thus, probability for a transfer of electrons from activated chlorophyll to molecular



**FIGURE 42.9** ROS production and detoxication. Detoxication of ROS by sequential ascorbate and glutathione cycles will consume NADPH and, thus, result in a relief of  $NADP^+$  shortage in high light. A prerequisite is that (1) enzymes involved are available at ample concentrations and (2) are positioned in ideal neighborhood to allow high turnover rates.

oxygen to form  $O_2^{\cdot -}$  will increase.  $O_2^{\cdot -}$  will rapidly dismutate to yield  $O_2$  and the less reactive ROS  $H_2O_2$ . But in the presence of some cations, like Cu and Fe, for instance, highly reactive  $OH^{\cdot}$  may be formed (Imlay and Linn, 1988; Figure 42.9).

$H_2O_2$  is one of the most important secondary messengers in plant tissues, modulating effects of hormones and involved in developmental control of cells and tissues (van Breusegem and Dat, 2006; van Breusegem et al., 2008). It has been shown that mitogen-activated protein kinases (MAP kinases) are involved in transduction of  $H_2O_2$  signaling on cellular level (Pitschke and Hirt, 2009). Apparently, MAPK3 and MAPK6 are integrating stress signals that regulate stomatal development (Wang et al., 2008a,b). Moreover, in an earlier paper, Verslues et al. (2007) have shown that nucleoside diphosphate kinase 2 (NDPK2) can interact with salt stress signaling salt overlay sensitive 2 kinase as well as with catalase. Genes under the control of these pathways have to be identified. A mode of cell signal tuning may occur via regulation of transcript stability. In agreement with this assumption, it has already been found that stability of the SOS1 mRNA, induced under salt stress, is enhanced with increasing ROS concentration (Chung et al., 2008).

Ascorbic acid is a major antioxidant in plants. It detoxifies reactive oxygen species and maintains photosynthetic function (Figure 42.9). Through its ascorbate recycling function, dehydroascorbate reductase affects the level of foliar reactive oxygen species and photosynthetic activity during leaf development. As a consequence, this enzyme influences the rate of plant growth and leaf aging (Chen and Gallie, 2006).

ABA-induced closure of stomata and ABA-mediated inhibition of stomata opening are two ABA effects based on different reaction sequences (Mishra et al., 2006). Nevertheless, both processes



are fine-tuned by ROS signaling, and it appears to be clear that ROS signals are transduced via a cascade of MAP kinase reactions (Gudesblat et al., 2007).

#### 42.3.2.1.2 Detoxification of ROS

As stated above, ROS production occurs permanently at a certain probability. It is well documented that ROS as well as nitrogen radicals are involved in cell signaling (Wilken and Huchzermeyer, 1999) in plant cells like in cells of most other organisms. But, as high ROS concentrations are toxic, plants are equipped at varying degrees with several systems to detoxify ROS species. With this respect, molecules having antioxidative potential can be discriminated from enzyme-catalyzed reaction sequences.

Alpha-tocopherol is synthesized in the chloroplast (Schultz et al., 1976) and can be found in high concentrations in thylakoid membranes. Alpha-tocopherol disrupts lipid peroxidation cascades, reacts with  $O_2^-$ , and is capable of scavenging hydroxyl-, peroxy-, and alkoxy-radicals (Halliwell, 1987). Oxidation of alpha-tocopherol leads to formation of an alpha-chromoxyl radical, which can be reduced by ascorbic acid.

Ascorbic acid and several redox-active tri-peptides, called glutathiones, can be found in chloroplasts in millimolar concentrations (Halliwell, 1982). Several different types are known from plants (Schmidt and Jäger, 1992). This finding suggests that they are involved in different pathways controlled by specific enzymes. But there is only little information available to date.

In chloroplasts,  $H_2O_2$  can be detoxified by an ascorbate-specific peroxidase (Chen and Asada, 1989) involved in the ascorbate–glutathione cycle (Halliwell and Gutteridge, 1986) (Figure 42.9) while in the cytosol  $H_2O_2$  detoxification is catalyzed in a catalase-dependent reaction. Other enzymes involved in detoxification of ROS are superoxide dismutase, which converts  $O_2^-$  to  $H_2O_2$ , and several peroxidases (Chang et al., 1984).

Antioxidants as well as enzymes capable of detoxifying ROS are present in all plants and plant tissues. But their concentrations and catalytic activities, respectively, as well as their patterns differ a lot (Streenivasulu et al., 2000). Therefore plants differ in their capacities (1) to immediately detoxify ROS upon their occurrence and (2) to build up a detoxification potential under stress. If the balance between production of ROS and quenching capacity of the respective tissues is upset, oxidative damage will be produced (Harper and Harvey, 1978; Dhindsa and Matowe, 1981; Wise and Naylor, 1987; Spychalla and Desborough, 1990). In experimental approaches, it was demonstrated that (1) enzyme activities of antioxidative pathways increase as a salt stress response (Verma and Mishra, 2005) and that (2) the maximal level of salt tolerance correlated with maximal respective enzyme activities (Gossett et al., 1994; Hernandez et al., 1995, 2000; Sehmer et al., 1995; Kennedy and De Fillippis, 1999; Benavides et al., 2000; Lee et al., 2001; Mittova et al., 2002, 2003).

Their importance in developing salt stress tolerance of individual antioxidants along with enzymes stimulating their turnover has been documented in several approaches using molecular genetic techniques. The *Arabidopsis* *soz1* mutant contains only one-third of the ascorbate concentration of the wild type. As expected, the mutant is less tolerant toward ROS stress as compared to the wild type (Conklin et al., 1996). The observed positive correlation between a plant's antioxidative capacity and salt stress tolerance was supported further by analysis of salt stress–induced changes of mRNA patterns. In citrus, for instance, not only enzyme activities of Cu/Zn-SOD, glutathione peroxidase, and cytosolic APX increase, but also the respective mRNAs were found at higher abundance (Holland et al., 1993; Gueta-Dahan et al., 1997). This result suggested that plants are able to adapt their antioxidative capacity to physiological needs. Moreover, de novo synthesis of enzymes rather than regulation of those already present appears to be essential. This idea was supported by Wilkens et al., who used antisense mutations to produce catalase-deficient tobacco plants. They found that catalase deficiency results in ROS sensitivity when salt stress is applied (Wilkens et al., 1997).

In addition to the above-described negative controls, improving stress tolerance by overexpression or de novo implementation of genes has been tested as well. Successful approaches of this kind

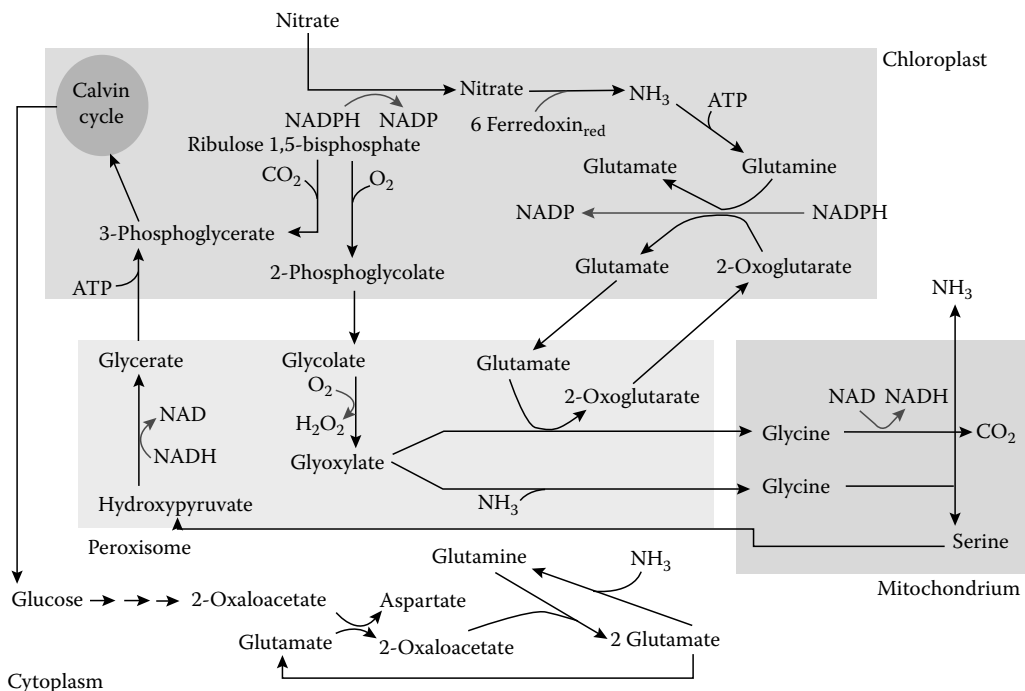
leading to improved stress tolerance increased the concentrations of (1) mitochondrial Mn-SOD, Fe-SOD, chloroplastic Cu/Zn-SOD, and glutathione-*S*-transferase/glutathione peroxidase (Bowler et al., 1991; Gupta et al., 1993a,b; Van Camp et al., 1996; Shikanai et al., 1998; Roxas et al., 2000) or (2) of Bet-A product leading to enhanced concentration of glycine betaine (Bhattacharya et al., 2004).

In summary, these results indicate that salt stress tolerance may be improved by overexpression of genes involved in the antioxidant system of plants. The advantage of this approach is that mostly only one or a very limited number of genes have to be overexpressed to improve scavenging of ROS.

A strategy to improve stress tolerance of plants might be to treat cultures with compounds stimulating the production of antioxidants or enzymes involved in detoxification pathways. Apparently, putrescine is such a compound. It was found that putrescine treatment of *Brassica* resulted in increased contents of glutathiones and carotenoids, and that this increased antioxidant content of leaves was paralleled by stimulated growth rates of mustard seedlings under stress (Verma and Mishra, 2005).

#### 42.3.2.2 Photorespiration

Closure of stomata thus inhibiting gas exchange is a secondary effect of salt stress, mostly brought about by ABA released from the plant roots. In the presence of light,  $O_2/CO_2$  ratio will increase inside leaves and impair  $CO_2$  fixation especially in  $C_3$  plants. This happens as the enzyme Rubisco can bind  $O_2$  instead of  $CO_2$  to its reaction center, thus catalyzing synthesis of a  $C_3$  plus a  $C_2$  compound instead of two  $C_3$  compounds in the primary reaction of the Calvin cycle (see Figure 42.10). The  $C_2$  compound, 2-phosphoglycolate, will be converted to glycolate, a molecule that cannot be metabolized by chloroplasts. Concentration of this molecule eventually would become toxic.



**FIGURE 42.10** Linking metabolic pathways of cell compartments. Biochemical pathways of chloroplasts, peroxisoms, and mitochondria are linked via shuttle systems. With respect to energy flow, photorespiration and nitrate reduction are of focal interest. Like the Calvin cycle, nitrate reduction is consuming electrons released from photosystem I. Thus, nitrate reduction is recycling the cofactors ferredoxin and NADP<sup>+</sup>. Photorespiration, on the other hand, is transferring redox power to the mitochondria and is involved in shuttling of ammonia.

Detoxification and recycling of the  $C_3$  compound 3-PGA are understood to be the major function of a pathway called photorespiration. Photorespiration takes place by metabolite transfer between three compartments: chloroplasts, peroxisomes, and mitochondria.

The photorespiratory reaction cycle we know from our textbooks is a simplification allowing general estimates. We know that amino acids (glycine, serine, glutamic acid, and glutamine) may be subtracted from or fed into the cycle *in vivo*. Such reactions modify nitrogen flow among cell compartments. But, with respect to equilibrium of carbon flow, another aspect may be more important: It has been shown by Niessen et al. that mitochondrial glycolate oxidation contributes to photorespiration in higher plants as well (Niessen et al., 2007). This finding not only complicates photorespiratory reaction mechanism, it also provides further options of fine-tuning of the system: (1) the mitochondrial glycolate dehydrogenase reaction contributes to energy balance of oxidative phosphorylation (Paul and Volcani, 1976); (2) the turnover capacity is lower as compared to the peroxisomal pathway. Thus the total energy balance of photorespiration will vary with *Rubisco* oxygenase activity.

Several efforts have been made to improve plant productivity. These include attempts to improve the efficiency of *Rubisco* by engineering or by prospecting known organisms for more efficient variants (Andrews and Whitney, 2003). The most ambitious approach would be to engineer  $C_4$  photosynthesis into a  $C_3$  cereal, because it requires targeting photosynthesis pathways in two different cell types. Moreover, it would include structural modification of leaf tissues. Therefore, it is not a surprise that no such experiments have been reported to be successful so far. But an impressive result has been presented by Kebeish et al. They managed to express a photorespiratory bypass inside chloroplasts (Kebeish et al., 2007). They took advantage of the glycolate catabolism from *E. coli* that is using  $NAD^+$  as an electron acceptor and does not produce ROS. Moreover, the bypass is releasing  $CO_2$  inside chloroplasts rather than inside mitochondria. This way refixation of  $CO_2$  is favored. Nevertheless, the success is stunning, because chloroplasts are not known to be capable of accumulating  $CO_2$ . Therefore, the advantage probably is due to improved turnover rates rather than enhanced substrate ( $CO_2$ ) concentrations. Such an interpretation would be in line with our arguments. On this basis, it may be concluded that salt stress can interfere with protective effects of photorespiration by inhibition of its turnover capacity; by intermediate shuttling among compartments, for instance.

It has to be kept in mind that photorespiration is a major source of  $H_2O_2$  in illuminated  $C_3$  leaves. On the other hand,  $H_2O_2$  production and interaction with pyridine nucleotide coenzymes make photorespiration an important player in cellular redox homeostasis. Furthermore,  $H_2O_2$  is an important second messenger controlling cell development and tuning effects of hormones like ABA, for instance. Any interference with this reaction will have effects not only on immediate energy status and phosphorylation efficiency, but also on plant hormonal responsiveness, thus on development and plant life cycle (Foyer et al., 2009).

In most textbooks, it is stated that photorespiration is a major problem of  $C_3$  plants rather than  $C_4$  and CAM plants. The latter ones can overcome the problem on the expense of extra energy consumption by using the  $C_4$  pathway, i.e., binding  $CO_2$  to PEP to form a  $C_4$  compound (that can be reduced to malate, for instance). In the final carbon fixation step,  $CO_2$  will be released from the  $C_4$  compound, and assimilation will be catalyzed by *Rubisco* in an environment characterized by low oxygen partial pressure.

Based on these findings, it is argued that formation of significant concentrations of glycolate is not a problem of  $C_4$ - and CAM plants. If salt stress would interfere with aggregate formation of chloroplasts, mitochondria, and peroxisomes, thus inhibiting by glycolate toxicity proper performance of  $C_3$  plants; such effects should not be observed in  $C_4$ - and CAM plants. Nevertheless, salt stress might interfere with intermediate transfer among cells and cell compartments in  $C_4$ - and CAM plants. This would inhibit photosynthetic activity but would not result in “classical” glycolate toxicity.

But, again the situation apparently is not as simple as suggested in textbooks. Several studies have pointed out that a low rate of photorespiration takes place in  $C_4$  plants as well. In maize leaves

grown at normal CO<sub>2</sub> concentration, photorespiration may reach 5% of the rate found in tobacco grown under identical conditions (Zelitch, 1973). Though such a rate may appear to be low, glycolate oxidase activity obviously is essential for C<sub>4</sub> plants. Maize plants showing less than 10% of glycolate oxidase activity of the wild type could grow at high CO<sub>2</sub> concentrations, they became necrotic in normal air, and died within 2 weeks (Zelitch et al., 2009).

There is evidence that glycolate can inhibit QA/QB electron transfer in PSII (Petrouleas et al., 1994). As stated above, this would lead to stimulated ROS production. This interpretation is in line with the observed bleaching of maize in presence of glycolate (s.a.). In several publications, it is suggested that the function of photorespiration is to serve as a sink to dissipate excess redox energy (Kozaki and Takebe, 1996; Winkler et al., 2000). In terms of our arguments, this would mean photorespiration is recycling coenzymes functioning as acceptors of photosynthetic electron transport chain. This would help preventing ROS production as well. But, to our understanding, this interpretation does not sufficiently take into account the compartmentation of coenzymes.

#### 42.3.2.3 Non-Photochemical Quenching of Energy

Under conditions that limit assimilation of CO<sub>2</sub> the potential rate of NADPH production exceeds the actual rate of consumption of reductive power. In order to be able to grow under stressful conditions, plants have to be equipped with mechanisms preventing excess reducing power. But, these futile mechanisms compete with photochemistry for absorbed energy. They lead to a decrease in quantum yield of photosystem II (Genty et al., 1989).

The photosystem II antenna is highly flexible in tuning delivery of excitation energy to the photosystem II reaction center (Horton et al., 1996). The principal adaptation mechanism in photosynthesis is the control of thermal dissipation of excess energy within the photosystem II antenna, thus matching physiological needs (Johnson et al., 2009). In C<sub>3</sub> plants, losses by this mechanism, named non-photochemical energy quenching, may exceed the ones caused by photorespiration. Despite extensive investigations, the reaction mechanism of photochemical quenching is not completely understood by now, because (1) turnover of intermediates is fast and (2) reaction depends on intact structures of protein complexes and their *in vivo* arrangement inside the thylakoid membranes; i.e., reaction partners may not be extracted and individually analyzed. But some insight was achieved by a combination of molecular biological and biophysical techniques (Johnson et al., 2009).

For experimental approaches investigating salt stress effects, it is important to know that non-photochemical quenching of excitation energy is comprised of a fast and a slow component, qE and qI, respectively. Both reactions are reversible. The trigger of qE is the ΔpH across the thylakoid membrane sensed by the PsbS subunit of the light-harvesting complex (Li et al., 2000, 2004). Full expression of qE is associated with the enzymatic de-epoxidation of violaxanthin to zeaxanthin. This reaction is part of the xanthophylls cycle (Havaux et al., 2007). Enzymes involved are pH controlled and function on the expense of NADPH (Demmig-Adams and Adams, 1996). This makes the cycle a futile reversible reaction sequence on its own. The majority of photoactive xanthophylls is bound to the light-harvesting complex. In addition to the ones involved in the xanthophyll cycle, lutein and lutein epoxide are bound there as well and can be turned over in a cycle on their own (Matsubara et al., 2001). Depending on distances among pigments, these two cycles can interact with soluble xanthophylls and control non-photochemical energy quenching in a synergistic, but not well-understood way (Johnson et al., 2009).

Sensitivity of non-photochemical quenching to any experimental approach interfering with membrane structure and protein fine structure indicates that this reaction sequence of outstanding physiological importance will be highly sensitive to any reaction causing imbalance of ionic homeostasis. The threshold ion concentration resulting in significant inhibition of non-photochemical quenching will depend on availability of compatible solutes, for instance. Based on current understanding, it can be expected that such adverse effects can be monitored (1) by measuring pigment shifts due to altered ratios of xanthophylls and (2) by high-resolution chlorophyll fluorescence measurement.

Thus, it should be possible to measure salt effects on non-photochemical quenching even in the field using noninvasive techniques.

Especially, near the end of life cycle of leaves, masking of chlorophyll by anthocyanins becomes important. It prevents photooxidative damage and allows an efficient nutrient retrieval from leaves to storage organs (Field et al., 2001). Some plants use such mechanisms regularly when under salt stress. They can be identified, because leaf color will vary depending on their growth conditions like it can be observed with *Salicornia* and *Sempervivum*, for instance.

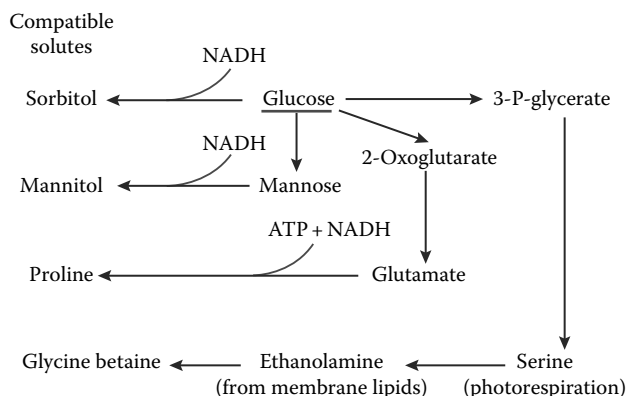
### 42.3.3 PROTECTING FROM DIRECT SALT EFFECTS ON PROTEIN AND MEMBRANE STRUCTURE AND FUNCTION

#### 42.3.3.1 Control of Mechanisms Improving Stress Tolerance: Compatible Solutes

As mentioned in the physiology section, sequestration of ions to the vacuole is a strategy leading to enhanced salt stress tolerance. This strategy requires compensation for osmotic and ionic potential of vacuolar ions. Part of the ionic component is delivered by surface charges of cytosolic proteins. As a rule, the Donan potential of proteins is beyond experimental access, and no reliable data are available to date (Gerendás and Sattelmacher, 2002).

Some low molecular weight, nontoxic compounds have been identified to significantly contribute to ionic and osmotic balance inside cells. They are called “compatible solutes” (Yancey et al., 1982; Ford, 1984; Ashihara et al., 1997; Hasegawa et al., 2000; Zhifang and Loescher, 2003). Chemically they can be described to be poly-amines or poly-hydroxyls. In addition to their function in ionic and osmotic homeostasis, their important function is that they can replace water in its function to stabilize aggregates of soluble proteins and membrane–protein interactions, respectively (Yancey et al., 1982; Crowe et al., 1992).

Depending on enzyme patterns and metabolic pathways preferred in individual plants, different compatible solutes have been found in plants (Figure 42.11). Among the compounds described in the literature are proline (Khatkar and Kuhad, 2000; Singh et al., 2000), glycine betaine (Rhodes and Hanson, 1993; Khan et al., 1998; Wang and Nil, 2000), sugars (Pilon-Smith et al., 1995; Bohnert and Jensen, 1996; Kerpesi and Galiba, 2000), di-, tri-saccharides and other sugar derived compounds (Hagemann and Murata, 2003), and polyols (Ford, 1984; Popp et al., 1985; Orthen et al., 1994; Bohnert et al., 1995). Overexpression of genes of metabolic pathways leading to production



**FIGURE 42.11** Overview on metabolism of compatible solutes. Glucose can function as a substrate for synthesis of compatible solutes. As a rule, cytosolic synthesis occurs at the expense of NADH. Thus, synthesis of compatible solutes is a relief under conditions, when production of redox power and glucose are exceeding consumption of electrons and export of sugar. Cytosolic pattern of compatible solutes varies among plant species depending on respective enzyme activities. In addition to regulation of respective gene expression, synthesis rates are controlled by availability of precursors.

of compatible solutes has been shown to improve salt tolerance. A prerequisite is that (1) substrates are available in ample amounts, and (2) overproduction of these compounds does not interfere with plant growth and development (Parida et al., 2002; Parida and Das, 2005).

Apparently, there are three ways to explain the mechanism how compatible solutes become overproduced under stress. Under optimal growth conditions, these compounds are produced at low rates; therefore, their concentration is found to be low. Therefore, the question is, whether overproduction under stress is due to activation of already existing enzymes or *de novo* synthesis of such enzymes.

It was observed that many stress conditions like drought and salt stress, for instance, initially inhibit export of products of photosynthesis from their source tissues. This will result in enhanced concentrations of primary products of the Calvin cycle in leaf cells. As shown by Koyro and Huchzermeyer (2005) the most compatible solutes derive from primary products of sugar metabolism. Therefore, increased concentrations of metabolites of photosynthesis will stimulate synthesis rate of compatible solutes. Such an increase will become significant, if metabolite concentrations under optimal growth conditions are below the  $K_m$  values of enzymes catalyzing the initial reactions of the pathways leading to compatible solutes. This interpretation agrees with the findings of Soussi et al. (1998), who attributed enhanced concentrations of proline and carbohydrates under salt stress in chick pea to damage of metabolic pathways rather than to protective mechanisms.

A second working hypothesis is based on findings of Süß et al. (1993). They found that enzymes of individual pathways in chloroplasts tend to form clusters. Such conditions would contradict free mobility of intermediates. Thus, substrate concentrations localized to catalytic centers of enzymes may significantly differ from respective bulk phase concentrations. Calvin cycle enzymes, for instance, tend to bind to one another and thus form aggregates of proteins. This allows substrates as well as products formed to be shuttled from one enzyme to the next one of the respective pathway. Süß postulated that modulation of pathways in this model is brought about by modulation of enzyme neighborhood. From analysis of internal signaling within cells, it is well documented that such modulations can be brought about by protein phosphorylation and de-phosphorylation, for instance. Indeed, such phosphorylation-dependent variations in protein–protein interactions have been observed by Allen and Horton, when analyzing protein localization in thylakoid membranes (Horton and Foyer, 1983; Pursiheimo et al., 2001; Allen, 2002). Moreover, effects of protein–protein interactions inside thylakoid membranes on the efficiency of primary reactions of photosynthesis have been analyzed in detail by the teams of Dilley and Huchzermeyer (Huchzermeyer and Löhr, 1984; Laszlo et al., 1984; Huchzermeyer and Willms, 1985; Löhr et al., 1985; Löhr and Huchzermeyer, 1985; Huchzermeyer et al., 1986; Allnutt et al., 1989).

A third model refers to the observation that salt stress response of plants has been found to be under the control of hormones (ABA, for instance) and second messengers (sugar signaling, for instance). This implies that modification of enzyme patterns will result in modified metabolic activity of cells and tissues under stress. Such argumentation would explain the above observations on the level of gene activities. But we have to take into account that it has been observed that binding sites of motor proteins of the cell skeleton are under hormonal control as well. Thus, hormone action can influence neighborhoods of proteins. This interpretation will link the ideas of model 2 and 3. A similar interpretation holds true for sugar signaling that is known to include regulation of protein phosphorylation. It is well known that protein phosphorylation status controls formation of protein aggregates.

These latter arguments may suggest not to discuss three different models of regulation of metabolic pathways leading to the synthesis of compatible solutes. It rather appears to us that hormone- and secondary messenger actions explain how formation of protein aggregates may be controlled in plants.

Biochemical reaction sequences leading to improved salt tolerance are likely to act synergistically (Iyengar and Reddy, 1996). The reactions include (1) synthesis of compatible solutes, (2) stimulation of antioxidative enzyme activities, and (3) modifications of the photosynthetic pathway.

#### 42.3.3.2 Polyols and Sugars

A considerable percentage of assimilated CO<sub>2</sub> is found in polyols. As there is an equilibrium between polyol and sugar metabolism, a possible role of polyols in carbon storage under stress has been discussed (Vernon et al., 1993; Klages et al., 1999; Sun et al., 1999). Among their functions in cell physiology and biochemistry are compatible solutes, low molecular weight chaperones, scavengers of reactive oxygen species (Smirnoff and Cumbes, 1989; Bohnert et al., 1995), and compounds contributing to osmotic adjustment in the cytosol (Yancey et al., 1982; Ford, 1984; Ashihara et al., 1997; Hasegawa et al., 2000; Zhifang and Loescher, 2003).

In celery, mannitol is synthesized by the action of the enzyme mannose-6-phosphate reductase (M6PR). Enhanced concentrations of this compatible solute is found under salt stress (Zhifang and Loescher, 2003). It was shown that M6PR from celery under the control of the CaMV35S promotor introduced into *Arabidopsis*, a plant not producing significant amounts of mannitol, significantly improves salt tolerance of mutants as compared to the wild type (Zhifang and Loescher, 2003). The mutants were able to grow, flower, and produce seeds in soil culture irrigated with up to 300 mM NaCl.

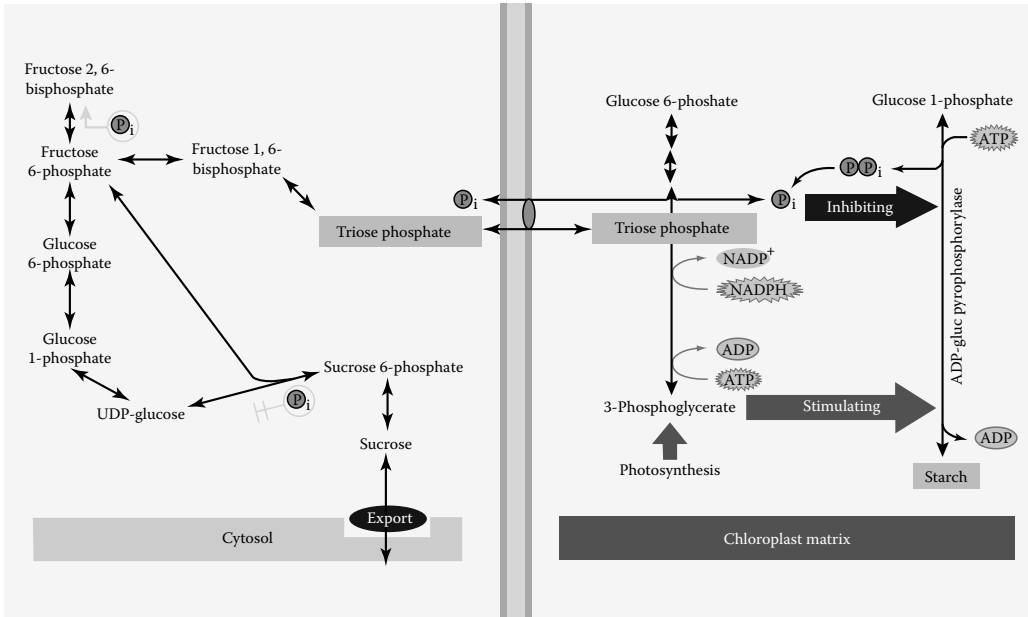
Pinitol is synthesized by the sequential action of inositol-o-methyltransferase and ononitol epimerase when significant concentrations of myo-inositol have built up (Bohnert and Jensen, 1996).

Sugars are synthesized in primary metabolism of plants. Therefore, bottlenecks in metabolic pathways and source-to-sink transport, respectively, translate to significant changes in sugar and starch concentrations in source and sink tissues. Sugar and starch concentrations may be used as indicators to localize cells, tissues, and plant organs most sensitive to salt stress. The problem in analysis of data from the literature is that in some papers data on sugar concentrations are not clearly aligned to specific cells or tissues but to “plant material,” roots or shoots. Moreover, not individual sugars are identified but “total sugar,” “soluble sugars,” “reducing sugars,” etc. Such information is of limited value, when it comes to analysis of the mechanism of salt stress effects on plant metabolism. However, relevant papers may help identify a promising experimental plant for analysis of a special salt stress effect on regulation of sugar metabolism.

Obviously, there can be a broad spectrum in sensitivity of enzymes and transporters of sugar metabolism within accessions of a species. It was found, for instance, that under salt stress sugar concentration in some genotypes of rice increase, while it decreases in other ones (Alamgir and Ali, 1999). Moreover, salt stress obviously is targeting several enzymes and transporters. Though concentrations of sugars vary in the shoot, starch concentration was observed to be constant in the shoot, while it was reduced with increasing salt concentration in rice roots. There are several options to explain stress responsiveness of root starch concentration. But a final conclusion would require data on enzyme activities and their metabolite concentrations available. As rice is one of the most important crops, parallel analysis of respective data will be done soon. Then, promising targets for future breeding for stress tolerance can be identified. Such an analysis has been performed by Dubey and Singh. They found that under salt stress the sugar content and the activity of sucrose-phosphate synthase increased, whereas the activity of starch phosphorylase decreased (Dubey and Singh, 1999).

Similar investigations on other crops are reported in the literature. Like in rice, sugar concentrations have been found to increase in tomato leaves. Starch content of leaves was not affected by salt stress (Khavarinejad and Mostofi, 1998). Under similar experimental conditions, Gao et al. observed that the activity of sucrosephosphate synthase in leaves is increased while acid invertase is decreased under salt stress (Gao et al., 1998).

As can be seen from Figures 42.11 and 42.12, such experimental approaches allow some more detailed analysis, but for identification of target enzymes (target genes for breeding) the complex regulation of sugar metabolism requires to measure more data in parallel. Another problem becomes obvious when analyzing anatomy and physiological differentiation of cells from the base to the tip of monocot leaves. Especially from field-grown cereals, it is well known that leaves located near



**FIGURE 42.12** Regulation of sugar status inside the chloroplasts and in the cytosol. Levels of sugar metabolites are permanently monitored inside chloroplasts as well as in the cytosol of leaf cells. Phosphate is one of the most important ligands controlling starch and sucrose synthesis in chloroplast and the cytosol, respectively. Inside the chloroplasts, free phosphate is inhibiting the ADP-glucose pyrophosphorylase. In the cytosol, phosphate is stimulating fructose-6-phosphate 2-kinase, while it is inhibiting both fructose-2,6 bisphosphatase and sucrose-phosphate synthase. Thus, phosphate limitation (caused by competition for uptake with chloride, for instance) will result in an inhibition of sucrose synthesis in leaf cells. As a physiological response of chloride stress, an inhibition of sugar export via the phloem will be observed.

the top of the shoot contribute most of photosynthetic activity of a plant. Old lower leaves become senescent or function as storage organs (to sequester salt and wastes) rather than being active in photosynthesis.

Like in dicots, young but full expanded photosynthetically active leaves contain young cells with young plastids as well as old cells with mature chloroplasts (Heintze et al., 1990). These cells differ in compartmentation of metabolic pathways as well as in stress sensitivity. Other than in dicots, where young and mature cells form patches all over the leaves, young cells are found preferentially at the leaf base while mature cells are found at the leaf tip in monocots (Heintze et al., 1990). This makes our cereals ideal experimental plants for the investigation of stress effects at different developmental states of leaf cells. Methods for analysis of primary and secondary metabolite synthesis are described in the literature (Heintze et al., 1990). Moreover, compartmentation of amino acids and the activity of amino acid transport into the vacuoles has been analyzed (Homeyer et al., 1989). The methods in use allow to analyze activities of amino acid transporters as well as ATPase and PPase activities (Homeyer et al., 1989).

In summary, it can be stated that experimental results currently available clearly prove salt stress effects on metabolism and compartmentation of sugars. As a rule, increased sugar concentrations can be found in leaves as a salt stress response. But published data do not allow to identify the individual mechanisms improving salt tolerance of some accessions of plants and the bottle necks of less tolerant plants of the same species, respectively. The network of metabolic pathways and the various regulations of enzyme and transporter activities are too complex and the data sets too limited to immediately allow a direct approach. Moreover, there are three other aspects impairing



direct comparison of experimental data from different publications (such an approach would help building a broader database):

1. Experimental material is not sufficiently described in many papers. Especially the developmental stage of plant material would need more precise description in many papers: It has been shown in several papers that permanent salt stress results in retarded development (late flowering, for instance) of crops (Bourque et al., 1975; Fahnenstich et al., 2008). This observation requires, especially in long-term experiments, to compare plants of identical developmental stage and not plants of the same age. For definition of developmental stages, activity patterns of marker genes may be used, for instance.
2. Salt stress not simply inhibits enzyme activities. It has been described that an increase of leaf sugar concentration is an immediate stress response. But, if plants can tolerate the applied stress, sugar concentrations will level off again and eventually will reach values of control plants (Fahnenstich et al., 2008). The period during which plants show elevated leaf sugar concentrations depends on the level of stress applied and the degree of salt tolerance of the plant. Therefore, it is essential for the comparison of experimental data to exactly know the timing of the experimental approaches.
3. It is well known that stress tolerance as well as responsiveness of plants varies with the developmental stage of plants. Plants showing high salt tolerance can have only limited tolerance in seedling stage (Mangroves, for instance). Like stated under (1), it will be essential to establish molecular markers or protein (membrane receptor) patterns for definition of developmental stages of experimental plants.

#### 42.3.3.3 Nitrogen-Containing Compatible Solutes

Like stated for polyols and sugars, the pattern of specific nitrogen-containing compatible solutes that accumulate under salt stress varies with plants species. In addition to the functions mentioned for other compatible solutes, nitrogen-containing ones are of special importance for maintenance of cytosolic pH. Due to its effects on ATPase and PPase activities, ionic stress impairs pH homeostasis. Therefore, it appears to be logical that the capacity to accumulate high cytosolic concentrations of nitrogen-containing compatible osmolytes is quite common among salt-tolerant plants (Mansour, 2000). Moreover, as nitrate and chloride compete for uptake at root level, storage of surplus nitrogen in organic molecules in periods of low stress might be a strategy to better prepare for subsequent harsh conditions. Stored "organic nitrogen" may lead to a delayed stress response of highly salt-tolerant plants.

Especially, glycine betaine has been found to be the dominant compatible solute in highly salt-tolerant plants, named halophytes (Khan et al., 1998, 1999, 2000; Saneoka et al., 1999; Muthukumarasamy et al., 2000; Wang and Nil, 2000). Glycine betaine is synthesized from choline in a two-step reaction sequence catalyzed subsequently by choline monooxygenase and betainealdehyde dehydrogenase (Rhodes and Hanson, 1993). It may be postulated that salt tolerance of plants can be increased by a molecular genetic approach, if they have a sufficient potential to provide choline as a substrate. Indeed, it was reported by Sulpice and coworkers that transformation of plants with the *codA* gene, coding for choline oxidase, resulted in improved salt tolerance of the mutant (Sulpice et al., 2003). As expected, it was observed that the mutants accumulated enhanced cytosolic concentrations of glycine betaine. A similar positive result has been reported by Bhattacharya et al., who transferred the bacterial *betA* gene for the synthesis of glycine betaine to cabbage. The transformed plants showed higher tolerance to salt stress as compared to the wild type (Bhattacharya et al., 2004).

Several amino acids have been found to increase in concentration under salt stress. The most prominent one is proline (Parida et al., 2002), but also valine, isoleucine, and aspartic acid have been observed to increase in concentration in leaf parenchyma cells of cereals (Mattioni et al., 1997; Elshintinawy and Elshourbagy, 2001). On the other hand, cysteine, methionine, and arginine are

reported to reduce in concentration upon salt stress. This observation indicates that there have to be more aspects to be mentioned but increased buffer capacity and contribution to osmotic homeostasis in order to sufficiently explain the phenomenon of improve salt tolerance of plants.

It is common to most of the dominant nitrogen-containing compatible osmolytes that they are zwitter ions having no net electrical charge at physiological pH. Therefore, they do not impair with surface charge patterns of proteins. On the other hand, they will bind to incoming salt ions and will form several layers of zwitter ionic shells around the ions. This way, the ion radius will significantly increase resulting in a significant decrease of charge density of each of the ion complexes. As one major aspect of ion toxicity is believed to be due to competition of ions with enzymes for their hydrate shell, reduction of charge density (reduced capacity to tightly bind water molecules) will reduce the apparent toxic effect of incoming salt ions.

When analyzing salt-induced increase of cytosolic proline concentrations in more detail, it became obvious that the effect cannot sufficiently be explained by an increase of precursor concentration or allosteric effects but *de novo* synthesis of enzyme protein is involved. This makes proline effects contributing to salt tolerance an ideal target for a molecular genetic approach.

It was found that in wheat activity of the enzyme delta-1-pyrroline-5-carboxylate reductase, involved in proline synthesis, is enhanced under drought stress as well as ionic stress, whereas activity of the enzyme proline dehydrogenase, involved in proline degradation, is inhibited exclusively under salt stress (Mattioni et al., 1997). It may be concluded that *de novo* synthesis of proline is the dominant regulatory mechanism of cytosolic proline concentration in wheat leaf cells.

#### 42.3.3.4 Aspect of Energy Consumption by N- and S-Pathways

As a rule, in nonwoody plants nitrate is reduced in chloroplasts, while it is reduced in root cell plastids of trees. As mentioned above in the physiology chapter already, nitrate competes with chloride (due to similar radii of the hydrated ions) for uptake via specific translocators. These translocators are regulated via the plant's sugar pool and their activity thus matches the photosynthetic activity of source leaves. In this chapter, we focus on coupling of nitrate reduction to photosynthesis in chloroplasts of weeds.

About one-third of the electrons released by PSII finally will be used for nitrate reduction. Therefore, nitrate reduction is a major sink for electrons and sufficient N fertilization contributes to prevention of primary reactions of photosynthesis from over-reduction in high light.

Nitrate reduction and incorporation of reduced nitrogen into glutamate is shown in Figure 42.10. It is obvious that the first intermediates of nitrate reduction are toxic. Like in primary reactions of photosynthesis, building up of concentrations and occurrence of side reactions that eventually might be toxic for the cell are prohibited by fast turnover of products. Direct neighborhood of enzymes involved, i.e., prevention of prolonged diffusion or release of intermediates to the bulk phase, is an essential prerequisite for this reaction sequence. Again, we have to refer to the ideas of Süss et al. (1993).

It has been shown in the literature that affinity for nitrate uptake from the soil can be improved by means of a molecular biological approach (Matt et al., 2001; Wang et al., 2003b; Lillo, 2004). Also, nitrate fertilization helps to reduce salt stress effects (Syvertsen et al., 1989; Murillo-Amador et al., 2006). In principle, the same arguments hold true that have been discussed at the end of the above paragraph.

It is obvious that salt stress can have direct as well as indirect effects on nitrate reduction and amino acid biosynthesis. Direct effects mostly are due to competition among nitrate and chloride ions. Indirect effects are based on dependence of nitrate reduction on (1) electrons released by photosynthetic electron transport, (2) intact structure of enzymes and enzyme aggregates, and (3) dependence of amino acid biosynthesis on substrates that derive from sugar metabolism (i.e., sugar supply from photosynthesis). We have to keep in mind that sugars, sugar phosphates, and ROS are functioning as secondary messengers regulating expression of enzymes. Therefore, there will be another level of regulatory effects. Though a lot information on sugar signaling has been analyzed

and a list of participants has been outlined in pretty much detail, the complex pathway structure of second messenger signaling is not well characterized to date (Sheen et al., 1999; Gibson, 2000; Sheen, 2002; Baena-González and Sheen, 2008).

As nitrate reduction and subsequent amino acid biosynthesis depend on photosynthetic activity, there has to be some buffer capacity for intermediates to allow continuous metabolic activity of plants. In principle, there are two storage compartments in leaf cells: plastids and the vacuole. In storage organs, plastids can differentiate to protein storage compartments. In chloroplasts, only low amounts of storage proteins typical for storage plastids are found. But in some papers it is discussed whether the huge amounts of *Rubisco* accumulating in chloroplasts may function as storage proteins as well. In vacuoles, free amino acids and other low molecular weight molecules are stored rather than macro molecules like proteins. It has been shown that vacuoles from tissues active in photosynthesis can actively import amino acids at final concentrations several fold exceeding the ones in the cytosol (Homeyer et al., 1989). Amino acid import occurs at the expense of a transmembrane pH gradient. Both, v-Type ATPase and PPase of the tonoplast build this gradient at the expense of ATP and pyrophosphate, respectively (Homeyer et al., 1989). It is well known that activity of these two enzymes is regulated (Taiz, 1992; Niu et al., 1995; Davies, 1997; Zhang and Liu, 2002; Han et al., 2005; Park et al., 2005), and that amino acid content is affected by salt stress (Pahlich et al., 1983). But in-depth understanding of regulatory pathways and regulation of transport activities under salt stress is still missing. Regulation of gene expression under stress has been observed some 20 years ago already, but research in this field is continuing in order to elucidate the regulatory network (Narasimhan et al., 1991; Park et al., 2005).

In contrast to nitrogen that is found exclusively in the reduced form in organic molecules, sulfur occurs in metabolites of biological relevance in several redox states. Only a minor portion of the photosynthetic electrons are used for the sulfate reduction pathway. As outlined by Schmidt and Jaeger (Schmidt and Jaeger, 1992, and citations therein), sulfate reduction pathway in chloroplasts differs from the one in bacteria that had been identified earlier.

Inhibition of sulfur metabolism has major secondary effects, because SH bounds are essential for functioning of catalytic centers of many enzymes and reduced sulfur is found in coenzymes like CoA and liponic acid, for instance. Moreover, a pool of various glutathiones forms the dominant redox buffer of plant cells, and glutathiones are involved in detoxification of heavy metals and ROS. Therefore, enhanced salt stress tolerance of sulfur metabolism always goes along with improved metabolic activity and apparent tolerance of other essential functions as well.

#### 42.3.4 SALT EFFECTS ON METABOLITE TRANSPORT AND BIOENERGETICS OF CELLS

Products of photosynthesis, sugars, amino acids, and lipids, have to be exported out of the chloroplasts into the cytosol of host cells and further on to sink organs. Compartmentation of metabolic pathways has been investigated in detail during the last 40 years (Lunn, 2007). Nevertheless, most information is available on sugar transport.

##### 42.3.4.1 Sugar Phosphate Export Out of the Chloroplasts

In the presence of light, sugars are exported as triose phosphates (DHAP and GAP) in exchange of free phosphate via the phosphate translocator (Riesmeier et al., 1993)].  $V_{\max}$  of sugar phosphate export is too low to keep up with sugar phosphate synthesis under average light conditions. This would cause shortage of phosphate inside the chloroplast stroma, thus inhibiting primary reactions of photosynthesis and increasing the risk of ROS production. Starch production inside the chloroplast is a relief, because phosphate will be released and becomes available for the chloroplast coupling factor to produce ATP.  $V_{\max}$  of starch production has not been measured yet as physiological sugar concentrations are too low. The capacity to produce starch obviously is high enough to turn over any sugar concentration that might become available under physiological conditions. There is an equilibrium between starch synthesis and hydrolysis, respectively. Therefore, starch will be

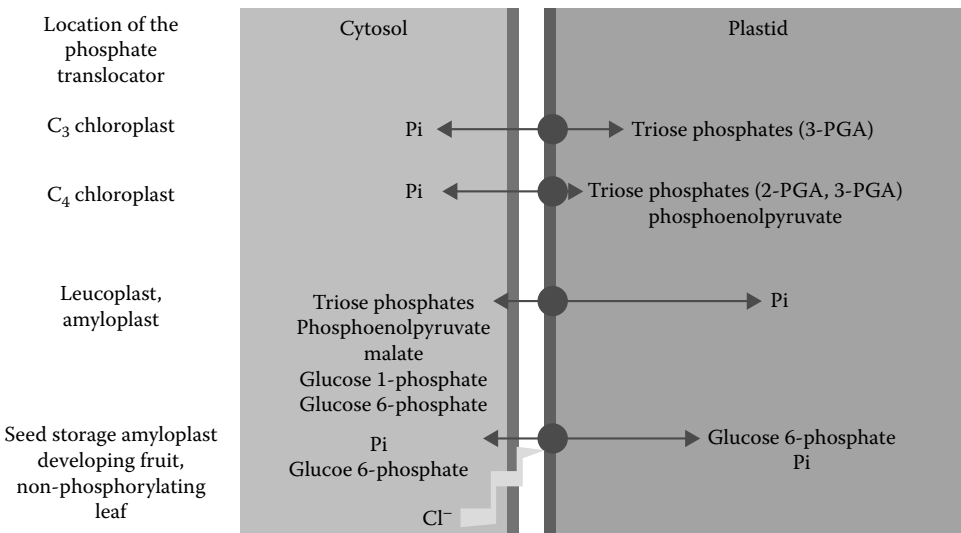
degraded at night and allow a permanent supplementation of the cytosol of the host cell. This system allows plant cells to grow permanently on a continuous flow of incoming sugar phosphates.

In the cytosol of green leaf parenchyma cells, sugar phosphates are used to fuel cell metabolism. In a competing metabolic pathway sucrose is formed from glucose and fructose and exported into the phloem to feed sink organs. As shown in Figure 42.12, there is a complex regulation of sugar metabolism inside and outside the chloroplasts, respectively. Moreover, with respect to the ideas of Süß et al., there will be a second level of regulation by aggregation of enzymes, not sufficiently analyzed to date (Adler et al., 1993; Süß et al., 1993). More reliable data are available concerning the function of hexose phosphates as second messengers tuning gene expression to the physiological and biochemical needs of an individual cell (Sheen, 2002 and citations therein).

From the above description it becomes clear that there are several alternatives, how incoming salt may affect functioning of cellular sugar metabolism. In principle, the situations in source and sink tissues are comparable. But, under moderate salt stress, salt concentrations have been found to differ a lot between root, leaf, and fruit tissues (see Sections 42.2.6.2 and 42.2.7). Therefore, the observed effect of salt stress also differs among tissues of plant organs.

Currently, salt stress effects on aggregation of enzymes to restructure neighborhoods and affect equilibria among biochemical pathways are poorly investigated. But it has been documented that product patterns of secondary metabolism varies a lot under stress (Zuther et al., 2007). As mentioned above, compatible solutes are stabilizing enzyme aggregates thus compensating salt effects at least to some extent. This matter will be analyzed in more detail as soon as reliable methods will be available for screening for such effects.

Salt effects on sugar metabolism and sugar export from the chloroplasts into the cytosol can be explained on the basis of a competition between  $\text{Cl}^-$  and  $\text{H}_2\text{PO}_4^-$  for binding sites on enzymes and receptors, respectively. This competition is due to similar diameters of these two ions when hydrated. From data currently available, it appears to us that the phosphate translocator is more chloride sensitive as compared to the other phosphate binding sites shown in Figure 42.13. This would mean that under salt stress the chloroplast stroma is at risk to run out of free phosphate. As mentioned above, this would inhibit ATP synthesis at the chloroplast coupling factor, which has an  $K$  of about 5 mM for phosphate (Groth et al., 2000). The observed effect would be quite



**FIGURE 42.13** Specificity of phosphate translocators. Phosphate translocators of plastidal inner envelop membranes differ in their specificity toward sugars transported in exchange of phosphate. It may be expected that  $\text{Cl}^-$  competes with phosphate at any of these translocators. This interpretation explains the observation that inhibition of sugar export from plastids is an early response under salt stress.

similar to the one observed after energy transfer inhibitors like Nitrofen (Huchzermeyer and Löhr, 1990). Nitrofen is a herbicide used in rice cultures and it is known to stimulate light-dependent ROS production in plants. By the way, this observation again is a proof for the tight coupling between primary reactions of photosynthesis.

With respect to reducing stress effects at a given salt concentration, thus improving crop yield, phosphate fertilization should help. On the other hand, stress tolerance of plant species could be improved if phosphate uptake at higher rate or higher specificity could be engineered by molecular biological methods. Any method preventing chloride import into photosynthetic active tissues would have a similar effect, of course. This consideration provides a good example demonstrating that it is not easy to decide how salt stress and stress relief, respectively, are acting on molecular level.

## 42.4 SUMMARY

The need to feed a fast-growing world population calls for ever-increasing food production. But increase of agricultural productivity is leveling off and per capita cereal production, therefore, is reducing year by year since the early 1990s. An alternative would be to tap areas currently not used for agriculture, salt contaminated soils, for instance. But such an approach calls for information on plant performance under salt stress in order to be in the position to design appropriate cropping and breeding strategies.

In the first part of this review, we have summarized information on plant performance under salt stress. Halophytes, plants of the coastal ecosystems, can teach us strategies how to survive and finish life cycle under salt stress. As a rule, enzymes and cell organelles isolated from halophyte tissues are salt sensitive, similar to samples isolated from our regular salt-sensitive crops. Halophytes have developed strategies to avoid uptake of excess salt or to sequester salt from the cytosol. Moreover, halophytes are able to keep ion homeostasis and to regulate nutrient and water relations.

In the second part of this presentation, we focus on biochemical aspects, i.e., the basis of all physiological observations. As photosynthesis is a prerequisite for biomass production, we concentrate on information related to this essential sequence of reactions. After some 40 years of research, it is not possible to present information on all aspects treated in the literature. Another reason to concentrate on salt stress effects on photosynthesis relates to applied aspects: Salt stress-induced reduction of photosynthetic efficiency directly relates to several biochemical “signals” that easily can be monitored like chlorophyll fluorescence, for instance. A better understanding of plant metabolism under salt stress will allow to design monitoring systems to predict crop yield or to identify promising crop accessions at an early stage of their development. Last, but not least, we should mention that identification of enzymes that are under stress the bottlenecks of metabolic pathways, means to identify targets for salt-resistance breeding.

## 42.5 FUTURE PERSPECTIVE

Several papers recently have shown that accessions of plants species selected from environments differing in environmental stress differ in their tolerance. Marker-assisted selection of promising accessions, therefore, can be a promising approach to identify stress-tolerance strategies of individual plant species (Tuberosa et al., 2002). Such an approach will lead to a better understanding of physiological and biochemical needs thus allowing to design strategies to engineer salt stress tolerance of individual plant species.

Induced resistance of plants (IR) has been first described in the context of pathogen resistance. Subsequent to an infection, plants can develop enhanced resistance to a broad spectrum of pathogens. It was found that this type of resistance is under the control of the hormone salicylic acid (Ryals et al., 1996; Durrant and Dong, 2004). This type of resistance also can be induced by some natural or synthetic compounds. These compounds apparently induce a general stress resistance in

plants (Janda et al., 1999; Senaratna et al., 2000; Kohler et al., 2002). In general, it can be found that plant cells and tissues respond faster and develop a broad spectrum of stress response reactions. The basis of this latent capacity to react in *Arabidopsis* has been identified by Beckers et al. They found that occurrence of chemically induced resistance correlates with enhanced cellular concentrations of inactive proteins of MPKs, especially of MPK3 and MPK6 as well as the respective mRNAs (Beckers et al., 2009).

It has been found in experiments with grape (*Vitis vinifera*) as well as *Arabidopsis thaliana* that ROS release is among the first detectable signals subsequent to application of abiotic stress. It may be assumed that like in animal tissues  $H_2O_2$  is functioning as a signaling molecule leading to downstream responses (Mittler et al., 2004). Currently, it is an unanswered question, whether NO signaling in plants is capable of neutralizing  $H_2O_2$  signals like it has been observed in animal neurons (see for reference: Wilken and Huchzermeyer, 1999). The activation of two mitogen-activated protein kinases, MPK3 and MPK6, has been found to be essential for signal transduction in response to  $H_2O_2$  in *Arabidopsis* (Kovtun et al., 2000). Moreover, Rentel and Knight could show that OXII kinase acts as a  $H_2O_2$  sensor and activates MPK3 and MPK6 (Rentel and Knight, 2004).

Skopelitis et al. have drawn our attention to another aspect (Skopelitis et al., 2006). Under continuous salt stress, increased proteolytic activity is found especially in mature tissues. Degradation of proteins will produce enhanced cytosolic ammonia concentrations that may reach toxic levels if not efficiently removed (Lutts et al., 1999). In plant cells, the mayor pathway for ammonium detoxification is the GS/GOGAT system leading to the production of glutamine and glutamate (Lea and Mifflin, 1974). In addition, glutamate dehydrogenase, an enzyme abundant in plant tissues, can show aminating activity and catalyze reductive amination of 2-oxoglutarate. It has been shown that abiotic stress induced enhanced cytosolic ammonium concentration results in enhanced GDH activity (Lutts et al., 1999; Hoai et al., 2003). In salt-tolerant rice cultivars, GDH activity was found to increase subsequent to stress, and decreased in salt-sensitive rice (Kumar et al., 2000). These results indicate that GDH is a salt (abiotic) stress responsive protein involved in ammonia detoxification under stress. Indeed, it could be shown in experiments with vine and tobacco that GDH gene expression can be induced by ammonium ions as well as ROS that were generated as a salt stress response (Hassan and Fridovich, 1979). In more detailed analysis of salt-stress response of *Nicotiana tabacum*, it was found that in addition to GDH genes, isocitrate dehydrogenase genes were up-regulated as well (Skopelitis et al., 2006). This means that 2-oxoglutarate production will be enhanced under salt stress as well. In *in vitro* experiments with cell and callus cultures, it could be shown that addition of ascorbate interferes with salt-induced gene regulation. (Skopelitis et al., 2006). This may indicate that ROS are functioning as signaling molecules.

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# 43 Role of *Acacia ampliceps* in Managing Salt-Affected Lands

Nico Marcar, Shoaib Ismail, Arunee Yuvaniyama,  
and Raziuddin Ansari

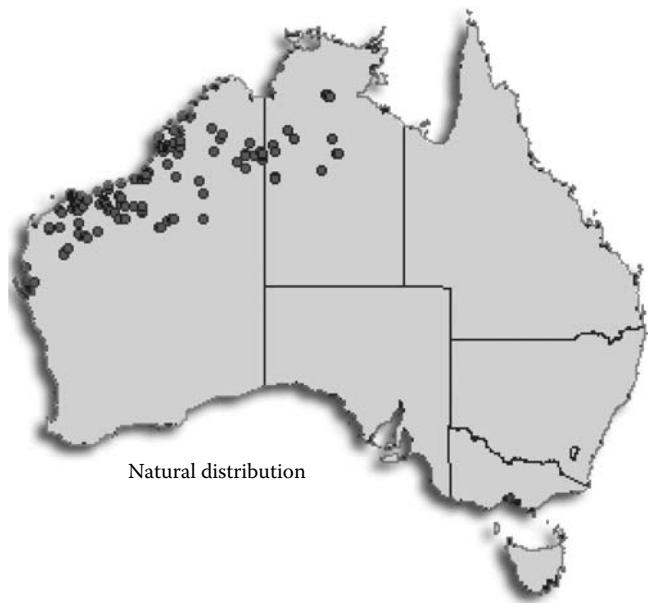
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## 43.1 DESCRIPTION AND NATURAL DISTRIBUTION

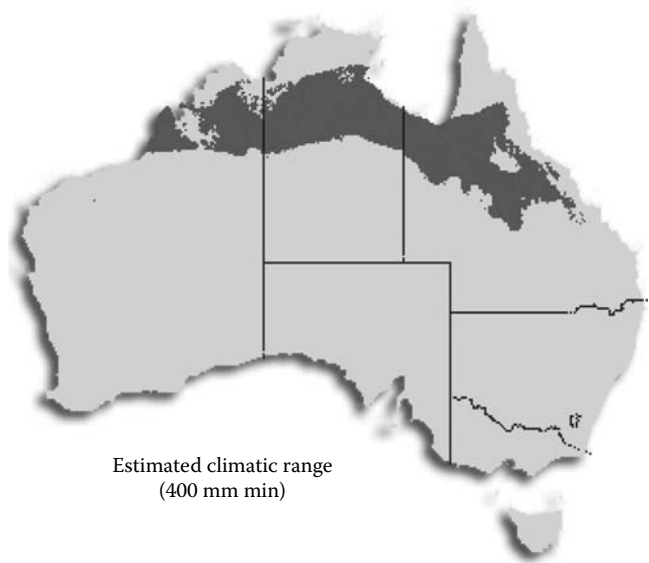
*Acacia ampliceps* Maslin. (commonly called salt wattle, *jila jila*, *nyarlka* [Australia]; Australian “katin” [Thailand]; Australian “kikar” [Pakistan]) is a large dense shrub or small, relatively short-lived tree (2–9 m tall) with one or multiple stems and a spreading canopy composed of branchlets that are often pendulous and yellowish. The bark is rough gray-brown for 1–2 m from the base, becoming smooth light green-brownish. Phyllodes are linear to lanceolate (7–25 cm long and 0.7–3 cm wide), shiny green, with a prominent yellow midrib. The globular flowers are white or cream, and appear in May to August in Australia (October in Thailand; January–February in UAE; February–March in Pakistan). Pods are hard, gray-brown (7–10 cm long and 0.5 cm wide), slightly constricted between the seeds, maturing in August to November in Australia (March–May in Thailand; June–July in UAE; July–August in Pakistan). Seeds are gray to black with a red aril. There are about 35 viable seeds per gram. More detailed descriptions are provided by Doran and Turnbull (1997) and Chapman and Maslin (2001). Flowering and seed production are normally prolific in the first 1–2 years after planting in Thailand and Pakistan (Marcar et al. 1998, Yuvaniyama 2009).

*A. ampliceps* occurs naturally along the coast and scattered localities in the warm to hot semiarid and arid zones of northwestern Western Australia and the Northern Territory (Doran and Turnbull 1997; Figure 43.1), but based on climate indices it can potentially grow well over a larger area in Australia (Figure 43.2). The long-term mean maximum temperature of the hottest month ranges from 32°C to 42°C and that of the coolest month from 11°C to 14°C (Marcar and Crawford 2004). Rainfall occurs mainly in summer with a long-term mean annual amount of 200–900 mm and 6–11 months of dry season.



Natural distribution

**FIGURE 43.1** Natural distribution of *A. amplexes*. (From Marcar, N.E. and Crawford, D.F., *Trees for Saline Landscapes*, RIRDC, Canberra, Australia, 2004, p. 146. With permission.)



Estimated climatic range  
(400 mm min)

**FIGURE 43.2** Potentially suitable areas for good growth of *A. amplexes* based on climatic indices. (From Marcar, N.E. and Crawford, D.F., *Trees for Saline Landscapes*, RIRDC, Canberra, Australia, 2004, p. 146. With permission.)

Near the coast, *A. amplexes* is found on plains and sandy dunes, while inland it is found on sandy plains and flood plains and along drainage lines or low-lying plains among rough hill country or low hilly tracts. It also grows very close to the tidal zone and in and around salt lakes. Soils are chiefly alluvial, either sandy or clayey and often alkaline. Soil types include calcareous earths, gray loams, red earthy sands, and cracking clays (Doran and Turnbull 1997). Occasionally

it is found in chenopod shrub land, acacia-dominated open-shrub land or, less frequently, in open woodlands. The best growth occurs on alkaline soils, which are free draining but with access to plentiful groundwater, especially where this is relatively shallow (2–3 m below the surface). Though it is often found in locations that receive run-on water, it is sensitive to periodic waterlogging and inundation. It does not tolerate acid soils.

## 43.2 INTRODUCTION AND KEY FEATURES

In Pakistan and Thailand, *A. ampliceps* was introduced along with other tree species two decades ago through projects supported by the Australian Center for International Agricultural Research (ACIAR), and subsequently it has been tested widely in the United Arab Emirates (UAE) and other countries in Asia/Middle East through several collaborative projects (Awan et al. 2007, ICBA 2007, 2009, Yuvaniyama et al. 2008). Key findings for *A. ampliceps* include the following:

- Grows well in moderately to highly saline and sodic soils though its growth is somewhat restricted by the presence of hard pans and under shallow water table and waterlogging conditions.
- Grows well on sandy, drought-prone soils as long as sufficient water is available.
- Relatively fast growing, producing abundant aboveground biomass.
- Coppices and root suckers, frequently producing clumps or dense stands.
- Establishes well by direct seeding on saline sites (e.g., at sites in Punjab, Pakistan; I Haq, personal communication). In northeastern Thailand, it has also been observed that seeds that have fallen onto the ground will germinate prolifically if subject to fire on fields used for growing rice (A. Yuvaniyama, unpublished data).
- Has a shallow root system when compared with other acacias such as *A. nilotica* (Marcar et al. 1998, Shah 2007).
- Copes with mild winter frosts (a few days at  $-1^{\circ}\text{C}$  to  $2^{\circ}\text{C}$ ; Marcar et al. 1998), some provenances continued slow growth even in the face of temperatures as low as  $-15^{\circ}\text{C}$ , when exposed for a few days (RETA 2008), frozen to the base at  $-3.8^{\circ}\text{C}$ , but continued sprouting from roots (Johnson 2004).
- Can be a host to the root hemiparasite, *Santalum album* (Indian sandalwood), which acquires nitrogen from it (Radomiljac et al. 1999). It is susceptible to insect attack in Australia; for example, from crusader bug, longicorn borer, and defoliating caterpillars (Doran and Turnbull 1997), and from arboreal cricket (Elliott et al. 1998).
- There is significant variation in survival, growth, form, and response to edaphic stresses including salinity, waterlogging, and frost among provenances and families (Marcar et al. 1998, 2003, RETA 2008).

Subsequently, the species is making steady inroads into public and private lands. It has the potential to be managed for multiple uses (e.g., fodder, fuelwood, timber) in mixed agricultural systems and to improve salt-affected land.

## 43.3 PRODUCTIVITY AND WATER USE

### 43.3.1 ESTABLISHMENT AND SILVICULTURE

*A. ampliceps* seeds are usually soaked in hot water ( $90^{\circ}\text{C}$ ) for 1 min or a similar period to break the seed coat, prior to germination in a tray or similar facility. After several days or when about 5 cm tall, seedlings are transferred into plastic bags in a nursery or else seeds are directly sown into plastic bags. Seedling bags are best placed on a flat, preferably well-drained surface, and ideally turned or moved regularly in order to prevent roots growing out of the bag. When plants reach about

30 cm (before they become too large), they should be carefully transplanted to the field; the plastic bag should be cut at the bottom or completely removed to allow roots to grow into the new soil. Anecdotal evidence based on poor root growth of *A. ampliceps* and other species established on saline land suggests that it is better to completely remove the bag rather than to partially remove or cut the bags at the base in an attempt to protect the young seedling from salts in the root environment (N. Marcar, unpublished data).

Two studies have demonstrated the beneficial effects of soil amendments (including rice husks and farm yard manure) and mulches in improving survival and early growth of *A. ampliceps* when soils are infertile as well as saline. Marcar et al. (1991a,b) reported on the effect of various treatments (e.g., mulching, soil amendments, fertilizer treatment) on the survival and growth at 6 months on a highly saline-sodic site in northeastern Thailand. Best survival and growth was found for plots treated with mulch, gypsum, and either nitrogen (N) and phosphorus (P) or farm yard manure; this effect was still evident after 57 months (N. Marcar, unpublished data). Ansari et al. (2001) reported on an experiment to investigate the effects of single and combined application of wheat straw mulch, NP fertilizer, and supplemental irrigation on survival and growth of *A. ampliceps*, *A. nilotica*, and *C. lancifolius* on a highly saline, waterlogging-prone site near Hyderabad, Pakistan. Mulch application was the only treatment to significantly improve survival, and none of the treatments improved growth.

Inoculation with salt-tolerant rhizobia may improve N-fixation and hence growth under saline conditions (Zou et al. 1995). Shirazi et al. (2001) reported that inoculation of *A. ampliceps* seedlings with specific salt-tolerant rhizobial strains (especially, strain 63/1, originally isolated from a natural stand of *A. ampliceps* trees near Lake Nongra, Northern Territory) improved survival and growth of *A. ampliceps* and *A. stenophylla* at a saline site near Tando Jam (Pakistan); however, growth was best when only 100 mg N kg<sup>-1</sup> of soil was applied. In a glasshouse experiment, Zou et al. (1995) showed that N fixation and growth of salt-treated *A. ampliceps* seedlings was improved when seedlings were inoculated with a salt-tolerant (63/1) rather than a salt-sensitive strain. On a highly saline site (EC<sub>e</sub> 11–25 dS m<sup>-1</sup>, pH 6.9–7.3) in northeastern Thailand, height and diameter growth were improved above those of control plots when *A. ampliceps* seedlings were inoculated with rhizobia, but only when rice husk mulch and gypsum were also supplied; when plants were only inoculated with rhizobia or combined with fertilizer or gypsum alone treatments, impacts were not statistically significant (Anuluxtipan and Pongwichien, unpublished data).

### 43.3.2 RESPONSE TO SALINITY: SURVIVAL AND GROWTH

#### 43.3.2.1 Field

*A. ampliceps* grows well on lighter-textured, neutral to very alkaline/sodic (pH 7–11) and highly saline (EC<sub>e</sub> up to 20–30 dS m<sup>-1</sup>) soils (Hussain and Gul 1991, McKinnell and Harisetijono 1991, Ansari et al. 1993, 1998, Marcar et al. 1998, 2003). Key results from available trials and demonstrations are summarized in Table 43.1. Species such as *A. stenophylla* (indigenous to Australia) and *A. nilotica* (indigenous to Pakistan) perform better under conditions of periodic waterlogging and flooding (Marcar et al. 2003).

In a field trial of five acacia species and *Atriplex lentiformis* near Tando Jam (Pakistan), *A. ampliceps* had the best survival and the next best growth after *A. nilotica* up to 3 years after planting (Ashraf et al. 2006). At EC<sub>e</sub> 4–12 dS m<sup>-1</sup>, survival was 80%–90% of nonsaline conditions and this decreased to about 50% at EC<sub>e</sub> 12–16 dS m<sup>-1</sup>. As part of a demonstration tree planting on 25 ha of highly saline (EC<sub>e</sub> 20–30 dS m<sup>-1</sup>) land near Badin, southern Pakistan, *A. ampliceps* had 55% survival, which was lower than that of *E. camaldulensis* (70%) and *A. stenophylla* (80%), probably because of the presence of a shallow, saline water table, which may have resulted in temporary waterlogging (Shirazi et al. 2006).

**TABLE 43.1**  
**Survival and Growth for *A. ampliceps* on Salt-Affected Land from Various Sources**

| Location                            | Mean Annual Rainfall (mm) | Site Factors                                 |                           |                                              | Management                                                 | Survival (%)                      | Height (m)          | Stem Diameter (DBH) (m) | Reference             |
|-------------------------------------|---------------------------|----------------------------------------------|---------------------------|----------------------------------------------|------------------------------------------------------------|-----------------------------------|---------------------|-------------------------|-----------------------|
|                                     |                           | Salinity (dS m <sup>-1</sup> )               | Sodicity ESP <sup>a</sup> | Other                                        |                                                            |                                   |                     |                         |                       |
| Hyderabad, Pakistan                 | 200–300                   | EC <sub>e</sub> <sup>b</sup> 7–26; 16 (mean) | 20–100                    | Loamy clay to silty clay                     | 2,500 trees ha <sup>-1</sup>                               | 75 (2 years)                      | 3.8 (3 years)       | 16.3 (3 years)          | Ashraf et al. (2006)  |
| Badin, Pakistan                     | 200–300                   | EC <sub>e</sub> 20–30                        |                           |                                              | >4,000 trees ha <sup>-1</sup>                              | 55                                |                     |                         | Shirazi et al. (2006) |
| Dubai, UAE                          | N/A                       | EC <sub>iw</sub> 30–35                       | —                         | Sandy, nonsaline                             | 1,100 trees ha <sup>-1</sup><br>(3 m × 3 m spacing)        | 95                                |                     |                         | ICBA (2006, 2007)     |
| Khorat, NE Thailand                 | 700                       | EC <sub>e</sub> 8.6                          | 17                        | Loam                                         | —                                                          |                                   | 10 (10 years)       |                         | Yuvaniyama (2009)     |
| Hyderabad, Pakistan                 | 200–300                   | EC <sub>e</sub> 5–40 (mostly > 15)           | 10–15                     | Silty to clay loam                           | 2,500 trees ha <sup>-1</sup>                               | 27, 49 (3 years)                  | 4.3, 4.7 (3 years)  | 7.1, 8.0 (3 years)      | Ansari et al. (2001)  |
| Hyderabad, Pakistan                 | 200–300                   | EC <sub>e</sub> 5–40; 21 (mean)              | 10–15                     | Silty to clay loam; inundation after 2 years | 2,500 trees ha <sup>-1</sup>                               | 78–100 (21 months); 0–7 (3 years) | 2.9–3.3 (21 months) | 4.2–7.8 (21 months)     | Marcar et al. (2003)  |
| Pacca Anna via Faisalabad, Pakistan | ~500                      | EC <sub>e</sub> 5–15                         | (pH 8–9)                  | Silty clay loam; provenance-family trial     | 3 m × 2 m spacing; irrigation water (EC <sub>iw</sub> 4–6) | 89–96 (28 months)                 | 2.9–3.9 (28 months) |                         | Marcar et al. (1998)  |

<sup>a</sup> Exchangeable sodium percentage.  
<sup>b</sup> Electrical conductivity of the extract of a saturated soil paste.

*A. ampliceps* has grown well with a 40%–60% survival on sites in northern Queensland with  $EC_e$  up to  $20 \text{ dS m}^{-1}$  or higher (House et al. 1998). Under dry arid and semiarid conditions of the UAE, it has shown greater than 95% survival with a high growth rate on sandy soils, when irrigated with water of salinity ranging between  $EC_{iw}$  30 and  $35 \text{ dS m}^{-1}$  (ICBA 2006, 2007). In the Central Kyzylkum desert of Uzbekistan, *A. ampliceps* has been grown with drainage water salinities ranging between  $EC_{iw}$  12.5 and  $18.1 \text{ dS m}^{-1}$  (Toderich et al. 2009). *A. ampliceps* has survived well on moderately to highly saline soils in northeastern Thailand (Im-Erb et al. 2004), especially when grown on the bunds (mounds) surrounding rice paddies.

*A. ampliceps* does not perform well on acidic soils. For example, Karachi et al. (1997) reported that *A. ampliceps* did not survive up to 30 months on an acidic soil (pH 4.9) in Tanzania, in contrast with other Australian species including *A. auriculiformis*. Survival of *A. ampliceps* was poor when grown on acidic soils in northeastern Thailand (Anuluxtipan and Pongwicheng, unpublished data).

*A. ampliceps* has proven to be very resilient and productive when established on moderately to highly saline, sodic soils in Pakistan and Thailand. When *A. ampliceps* was planted on the bunds (mounds) surrounding salt-affected rice fields ( $EC_e$   $8.6 \text{ dS m}^{-1}$ , pH 8.7, ESP 17%) in northeastern Thailand, growth rates measured by increases in height and circumference (at 130 cm above ground) were moderately fast—about  $0.75 \text{ m year}^{-1}$  and  $1.0 \text{ cm year}^{-1}$ , respectively (Yuvaniyama 2009). In the presence of short-term or seasonal waterlogging and inundation, survival and growth would be expected to be lower than under well-drained conditions (Marcar et al. 2003). This is most likely related, at least in part, to its shallow root system, a feature that prevents it avoiding wet, surface soil conditions that often prevail on sodic sites during monsoonal periods or under excessive irrigation. Under these situations, species such as *A. stenophylla* and *A. nilotica* perform better. Breaking of the hard pan through mechanical drilling and/or cultivation would provide an opportunity for roots to extend deeper.

Growth of *A. ampliceps* is expected to be significantly reduced at root zone  $EC_e \sim 10 \text{ dS m}^{-1}$ , with reduced survival above about  $20 \text{ dS m}^{-1}$ , based on trials in Pakistan (Marcar et al. 1998, 2003, Ansari et al. 2001), Thailand (Marcar et al. 1991b), and Australia (House et al. 1998). Under these conditions, soils generally have higher clay contents and are subject to short periods or seasonal wetness or waterlogging. Inundation in the trial reported by Marcar et al. (2003) after 2 years resulted in almost complete death of *A. ampliceps* trees under all salinity conditions. However, significantly different results have been noted when *A. ampliceps* has been grown on lighter soils in the UAE, Jordan, Syria, Oman, and Tunisia, with greater than 95% survival even after 4 years at  $EC_e$  of  $10\text{--}25 \text{ dS m}^{-1}$ ; mortality was not related to salinity but rather to prolonged frost (Ismail et al. 2007). This may be related to the sandier, better drained soils that occur in this region. Under saline-acid conditions ( $EC_e$   $11\text{--}25 \text{ dS m}^{-1}$ ; pH 4.5–7.5) in northeastern Thailand, *A. ampliceps* has grown poorly with yellowing leaves a feature; nevertheless, *A. ampliceps* was able to survive when soils were less acidic but rainfall was relatively low (674 mm annual) and evaporation high (1519 mm annual) in this region (Anuluxtipan and Pongwicheng, unpublished data).

#### 43.3.2.2 Greenhouse

*A. ampliceps* grown at soil  $EC_e$   $21.7 \text{ dS m}^{-1}$  in a greenhouse had about 50% of control survival with shoot growth reduced by about 75% (Shah et al. 2006). Khan et al. (2009) further reported on root development in response to salinity and showed that root length and weight were markedly reduced at  $EC_e$   $21.7 \text{ dS m}^{-1}$ . At  $EC_e$  11.3 and  $21.7 \text{ dS m}^{-1}$ , increasing the watering frequency increased growth. Chaudhry and Hussain (1993) reported that height growth of *A. ampliceps* plants in soil-filled pots was decreased by about 50% when treated with 1.6% NaCl solution for 3 months after treatment with lower concentrations of NaCl, and there were no injury symptoms.

Mahmood (2007) reported that 50% growth reduction of *A. ampliceps* in soil-filled pots after 2 years occurred at  $EC_e \sim 40\text{--}50 \text{ dS m}^{-1}$  with significant reduction (5%–17% depending on the parameter) first occurring at  $10 \text{ dS m}^{-1}$ . Increasing salinity resulted in decreased plant water contents. Root dry weight was reduced slightly more than shoot dry weight. Sodicity also reduced growth with 50%

reduction occurring at about SAR 40, while significant reductions in growth (8%–30%) were found at SAR 20. In general,  $EC_e + SAR$  ( $20\text{ dS m}^{-1} + 50$  or  $30\text{ dS m}^{-1} + 40$ ) were the combined effects causing about 50% growth reduction.

Physiological investigations have shown that the ability of *A. ampliceps* to maintain reasonable growth when challenged with increasing salt concentrations is related to its maintenance of low foliar Na:K ratios (e.g., up to  $EC\ 95\text{ dS m}^{-1}$ , Craig et al. 1990) and low foliar Na and Cl concentrations (Marcar et al. 1991a). Yokota (2003) showed that *A. ampliceps* seedlings were the most tolerant of five tree species tested, and that although proline was accumulated in response to NaCl treatment, its concentration was not related to the degree of salt tolerance.

### 43.3.3 ABOVEGROUND BIOMASS PRODUCTION

A number of studies have reported on aboveground air-dry biomass (branches and leaves) yields for *A. ampliceps* under saline and nonsaline field conditions. Key results from available reports are summarized in Table 43.2. It is evident that *A. ampliceps* can produce abundant biomass, especially under irrigated conditions and even under highly saline conditions (ICBA 2007, Shah 2007). The proportion of leaf to branch and stem varies from about 20% to >50%.

*A. ampliceps* performs well under semiarid and dry conditions as long as adequate water is provided. When grown in sandy soils at Dubai (UAE) and irrigated with moderately to highly saline water, *A. ampliceps* yielded an aboveground air-dry biomass (below 1.5 m height only) of  $7.83\text{ kg tree}^{-1}$  after 3 years (ICBA 2006, 2007). In an agroforestry trial at the same site, when irrigated with saline water for 4 years, aboveground biomass of *A. ampliceps* decreased by up to 50% from  $EC_{iw}$   $10\text{--}30\text{ dS m}^{-1}$  and was about  $9\text{ t ha}^{-1}\text{ year}^{-1}$  at  $EC_{iw}$   $30\text{ dS m}^{-1}$  (soil  $EC_e$  about  $27.5\text{ dS m}^{-1}$ ), including  $3.9\text{ t ha}^{-1}\text{ year}^{-1}$  of stem and branches (ICBA 2006, 2007; Figure 43.3 and Table 43.1). Aref et al. (2003) reported very high biomass production under very dry conditions (50 mm rainfall) in Saudi Arabia with other acacia species, including *A. stenophylla*, performing poorly; this unexpected result may be due to availability of groundwater, though this was not referred to. Akhter et al. (2005) showed that the water use efficiency (total dry weight/total water use WUE) of lysimeter-grown *A. ampliceps* was high, suggesting that it was drought-tolerant employing a conservative water-use strategy with high biomass production and high WUE, making it suitable for dry areas. The total aboveground biomass attained by *A. ampliceps* in this study was higher than that mentioned by Banik and Bhosale (1999), which was  $3.9\text{ t ha}^{-1}\text{ year}^{-1}$  at 3.5 years. Neelam and Chopra (1988) report on the early growth of *A. ampliceps* in a trial of Australian acacias at an arid field site near Jodhpur (India). Along with *A. stenophylla*, *A. salicina*, *A. stenophylla*, and *A. mearnsii*, it had the best growth after 15 months.

### 43.3.4 GENOTYPIC VARIATION

Marked differences among *A. ampliceps* provenances and families were found for height, crown diameter and volume, and frost susceptibility observed in a trial established on moderately saline-sodic land ( $EC_e$  about  $5\text{--}15\text{ dS m}^{-1}$ ), but not subject to waterlogging, near Faisalabad, Pakistan (Marcar et al. 1998). For example, survival among provenances varied from 52% to 96% and height growth from 2.9 to 3.9 m after 28 months. The best growth was demonstrated for the Wave Hill (Northern Territory) and Halls Creek (Western Australia) provenances and poorest growth by the Karratha (Western Australia) provenance. These findings accord well with results from other trials in Pakistan (S. Ismail and R. Ahmad, unpublished data 1996, Marcar et al. 2003).

There were significant differences among families (but not among provenances) in susceptibility to frost (a few days during winter with temperatures of  $-1^\circ\text{C}$  to  $2^\circ\text{C}$ ) with frost damage ratings varying from 2.6 to 4.0 ( $1.0 = >75\%$  foliage killed;  $4.0 = <25\%$  foliage killed), and with the most sensitive trees within families rating at 1.0 (Marcar et al. 2003). The better performance of some provenances may have been related in part to their greater frost tolerance. Provenance



**TABLE 43.2**  
**Aboveground Biomass Production Values for *A. amplexes* from Various Sources**

| Location                      | Rainfall (mm)                 | Soil Conditions                                                    | Management                                                                                      | Harvest Age (Years) | Aboveground Biomass                      |                                       | Ratio Phyllode (Leaf) to Branch and Stem | Reference                |
|-------------------------------|-------------------------------|--------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------------|------------------------------------------|---------------------------------------|------------------------------------------|--------------------------|
|                               |                               |                                                                    |                                                                                                 |                     | kg Tree <sup>-1</sup> Year <sup>-1</sup> | t ha <sup>-1</sup> Year <sup>-1</sup> |                                          |                          |
| Hyderabad, Pakistan           | 200–300                       | EC <sub>e</sub> 15–20 dS m <sup>-1</sup>                           | 2000 (?)                                                                                        | 2                   | 3.8 <sup>a</sup>                         | 7.6 <sup>a</sup>                      | 0.67 (assumed)                           | Ansari, unpublished data |
| Shorkot, Pakistan             | N/A                           | EC <sub>e</sub> (?) 15.8 dS m <sup>-1</sup>                        | >4000 trees ha <sup>-1</sup>                                                                    | 1.7                 |                                          | 18 <sup>a</sup> (28 <sup>a,b</sup> )  | —                                        | Shah (2007)              |
| Dubai, UAE                    | N/A                           | Sandy, nonsaline                                                   | 1100 trees ha <sup>-1</sup> (3 m × 3 m spacing) irrigation water (EC 25–30 dS m <sup>-1</sup> ) | 3                   | 2.6 <sup>a,d</sup>                       | 2.8 <sup>a,d</sup>                    | —                                        | Ismail, unpublished data |
| Saudi Arabia                  | 50                            | Sandy loam                                                         | 625 trees ha <sup>-1</sup> ; nonirrigated                                                       | 4                   | 15.9 <sup>c</sup>                        | 9.9 <sup>c</sup>                      | 0.28                                     | Aref et al. (2003)       |
| Kolhapur, south-western India | 891–1400 (range over 3 years) | Sandy                                                              | 9305 trees ha <sup>-1</sup> ; nonirrigated and nonsaline                                        | 3.5                 | 0.36                                     | 3.3 <sup>c</sup>                      | 0.21                                     | Banik and Bhosale (1999) |
| Hyderabad, Pakistan           | 200–300                       | EC <sub>e</sub> 7–26 dS m <sup>-1</sup> ; loamy clay to silty clay | 2500 trees ha <sup>-1</sup> (2 × 2 m spacing)                                                   | 2                   | 91 <sup>e</sup>                          | —                                     | —                                        | Ashraf et al. (2006)     |

<sup>a</sup> Air-dried.

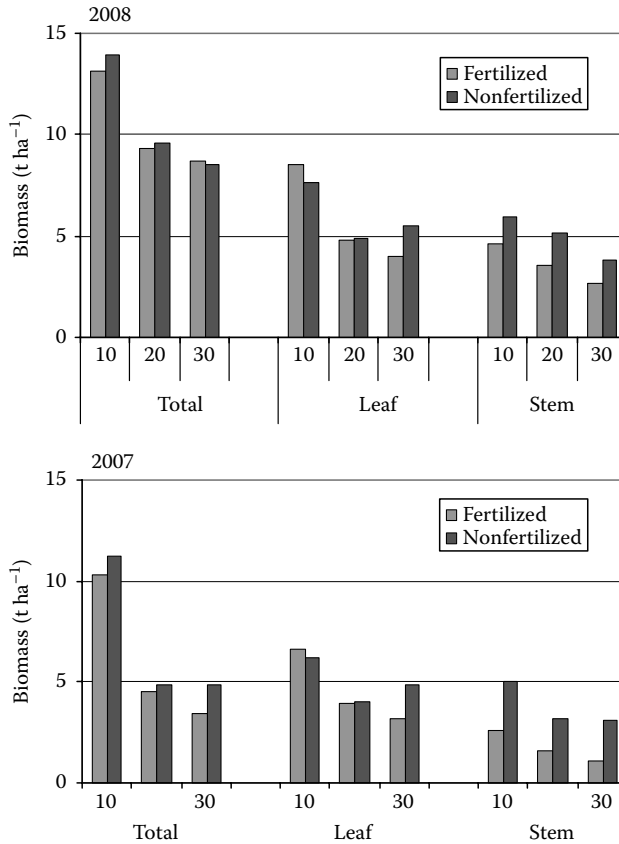
<sup>b</sup> Nonsaline conditions.

<sup>c</sup> Oven-dried.

<sup>d</sup> Trees were cut at 1.5 m to simulate grazing by goats; biomass production for the entire tree (up to 4 m in height would be greater).

<sup>e</sup> Green biomass.

*Note:* Data for single trees were normalized to an annual basis and extrapolated to a hectare basis using information on planting density.



**FIGURE 43.3** Aboveground biomass production for *A. ampliceps* irrigated with saline water ( $EC_{iw}$  of 10, 20, and 30 dS  $m^{-1}$ ) on a sandy soil in the UAE. (From Shoaib Ismail, unpublished data.)

variation for frost tolerance has also been demonstrated in trial plantings in Central Asia, Middle East, and in the Mediterranean region, with some provenances able to survive temperatures as low as  $-15^{\circ}C$  when exposed for a few days and to regenerate when temperatures become more favorable (ICBA-IFAD Report 2008, RETA 2008).

As a general rule, the faster-growing, highest biomass-yielding provenances are often less salt tolerant than slower-growing ones. For example, marked differences were found among seedlings of *A. ampliceps* provenances in response to irrigation with saline water ( $EC_{iw}$  10–40 dS  $m^{-1}$ ) for 1 year in studies conducted in Bangladesh, India, Pakistan, and UAE (Ismail and Dingel 2009). The Lake Dora (Western Australia) had the highest shoot and total biomass, but showed 50% reduction at  $EC_e \sim 15$  dS  $m^{-1}$  and zero yield at  $EC_e$  29 dS  $m^{-1}$ . The De Grey River (Western Australia) provenance was relatively low yielding but displayed high salt tolerance, with 50% reduction at  $EC_e$  25 dS  $m^{-1}$  and no survival at  $EC_e \sim 42$ –45 dS  $m^{-1}$ . Generally, field evidence for significant among-provenance response to salinity is less than that for absolute growth differences. However, some evidence for variation in response to root-zone soil salinity (measured with a hand-held electromagnetic induction device—EM38) among four provenances planted on highly saline site near Hyderabad (Pakistan) is available (Marcar et al. 2003). Results showed that the fastest growing provenances (Wave Hill [Northern Territory] and Hall's Creek [Western Australia]) were less tolerant (greater height reduction per unit of soil salinity) than the provenances from Karratha and Wolfe Creek Crater (Western Australia) after 21 months.

There is considerable scope to convert existing family-provenance trials (e.g., that reported by Marcar et al. 1998) to seed orchards, and new seed orchards to be established of the best performing provenances, for sustained supply of genetically superior seed. There is also a need to select for single

stem plants as against many stems from the ground level in most populations; evidence indicates considerable genetic component for this trait among individual trees within families (Marcar et al. 1998).

### 43.3.5 WATER USE

Water use of trees under different soil and climatic conditions will influence their ability to grow on and reclaim salt-affected soils, and utilize groundwater and/or scarce irrigation water resources. Only three studies (two in Pakistan and one in Thailand) have assessed transpiration rate of *A. ampicaps* under saline conditions using sap flow technology.

Khanzada et al. (1998) report on plantation water use of *A. nilotica*, *A. ampicaps*, and *Prosopis pallida*, watertable depth and soil conditions over 2 years near Hyderabad, Pakistan. On the highly saline site, annual water use (299 mm) by *A. ampicaps* was much less than that for *A. nilotica* (1248 mm), probably due to lower mean planting density and sapwood area (~500 and 1000 trees ha<sup>-1</sup>; 2.2 and 7.3 m<sup>2</sup> ha<sup>-1</sup>, respectively), the effects of partial waterlogging and inundation for 2 months, and their inability to access groundwater. Mahmood et al. (2001) reported on water use in a 3-year-old planting of *A. ampicaps* (1000 trees ha<sup>-1</sup> equivalent) at a moderately saline location near Faisalabad, Pakistan. While the basal area of *A. ampicaps* was similar to *E. camaldulensis*, its annual water use (624 mm) was less, probably in part because of high sensitivity of stomatal conductance to vapor pressure deficit and that it did not access groundwater. The consequences for salt accumulation in the root zone of *A. ampicaps* (and most tree species) over time (i.e., transpiration of water leaving the salt behind), while not shown to be excessive (e.g., Khanzada et al. 1998), needs continued assessment before recommending large-scale planting. Mettpranee (2004) measured transpiration in 5-year-old *A. ampicaps* trees in small plots (about 1000 trees ha<sup>-1</sup> equivalent) on a moderately saline-sodic, loamy sand soil in northeastern Thailand. Annual transpiration was about 545 mm, with higher values in the dry season than in the wet season.

In a pot lysimeter study, Akhter et al. (2005) showed that the water use efficiency (total dry matter/total water applied; WUE) of *A. ampicaps* was markedly better than that of *E. camaldulensis*, and confirmed its lower transpiration rate. *A. ampicaps* was shown to be drought-tolerant employing a conservative water-use strategy with high biomass production and high WUE, making it suitable for dry saline areas.

### 43.4 UTILIZATION

*A. ampicaps* has a role in sand dune stabilization, provision of low windbreaks, rehabilitating mine spoils, and for utilization and reclamation of saline land. Mahmood (2007) demonstrated significant reduction in soil sodicity and salinity after growing *A. ampicaps* for 5 years. In northeastern Thailand, rice yields have been shown to increase after growing *A. ampicaps* along the bunds of slightly to moderately saline paddy for a few years, and in addition, some areas could be recovered for growing rice again (S. Chomchan, personal communication 2009). Some severely salinized sites could be recovered to become plantations or forage areas when planting together with *Sporobolus virginicus* grass.

Although it has considerable potential as a fodder or forage, this has not been investigated in animal feeding trials. *A. ampicaps* foliage can, however, provide potential supplement for goats (Pakistan), sheep, and cattle (Craig et al. 1991; Qureshi and Barrett-Lennard 1998). Anecdotal evidence from Pakistan suggests that teddy goats find the shoots and phyllodes of *A. ampicaps* quite palatable, especially if left on the ground for a few days, and that no after effects occur from minor grazing (I. Haq, personal communication). It has also been found that cattle will eat cut foliage in northeastern Thailand (A. Yuwaniyama, unpublished data). There is potential for mixtures of *A. ampicaps* and halophytic shrubs and grasses in diets of livestock grazing on salt-affected land (Shah 2007). It also has potential for human nutrition since the seed is edible (Thomson 1992).

Table 43.3 summarizes some information from available sources about digestibility and nutritional content of *A. ampicaps* foliage. The digestibility (40%–55%) and protein contents (10%–15%) of

**TABLE 43.3**  
**Digestibility and Nutrient Analyses for *A. ampliceps***

| Predicted <i>in vitro</i> Dry Matter Digestibility (DMD) (%) | Dry Matter (%) | Crude Protein (%) | Crude Fiber (%) | Ash (%) | Gross Energy (MJ kg <sup>-1</sup> DM) | Ca (%)   | P (%)     | Reference                                                |
|--------------------------------------------------------------|----------------|-------------------|-----------------|---------|---------------------------------------|----------|-----------|----------------------------------------------------------|
| —                                                            | 22.0           | 10.3              | 19.6            | 20.7    | 18.4                                  | —        | —         | Khanum et al. (2007)                                     |
| 43.4–48.6                                                    | —              | 10.9–15.6         | —               | —       | —                                     | 2.6–3.4  | 0.08–0.12 | Vercoe (1992)                                            |
| —                                                            | 33.1           | 12.0              | —               | —       | 17.2                                  | 4.5, 5.1 | 0.08      | Trees grown on saline site; Yuvaniyama, unpublished data |
| 50–55                                                        | —              | 12–17             | —               | 11–15   | —                                     | —        | —         | Dynes and Schlink (2002)                                 |

foliage suggest this species is suitable for fodder at below maintenance requirements with the addition of some protein supplement. This relatively low dry matter digestibility is probably associated with the high lignin content. Khanum et al. (2007) reported that in vitro analysis of *A. ampicaps* leaves indicates quite acceptable values for metabolizable energy ( $7.6 \text{ MJ kg}^{-1} \text{ DM}$ ) and total digestibility ( $713.5 \text{ g kg}^{-1}$ ), among other analyses, and which compared favorably with values for leaves of *Sesbania aculeata* and *Leucaena leucocephala*, which are more conventional shrub forages. Crude protein (10.3%) was relatively low and ash content (20.7%) and crude fiber (19.6%) relatively high. They recommended *A. ampicaps* along with other salt-tolerant plants including *A. nilotica* as potential fodder plants.

Foliage nutritional composition of trees grown on salt-affected land will differ from those on soils with relatively well-balanced nutrient status. On slightly saline soil in southern Pakistan and northeastern Thailand, about 0.3% Na and about 1.5%–2.5% N were found in leaves (Shirazi et al. 2001, A. Yuvaniyama, unpublished data), whereas at high salinities Na concentrations were about 1%–2% and N concentrations ca. 1.7% (Marcar and Ansari, unpublished data; A. Yuvaniyama, unpublished data). Foliar concentrations of Na and Cl are considerably lower than those observed for halophytic shrubs; high salt loads in foliage present various problems for livestock health (Masters et al. 2007). Antinutrient compounds, such as tannins, may be present, but have not been investigated.

*A. ampicaps* wood is hard and tough, with potential for posts and small poles. Air-dry density has been recorded at  $625\text{--}770 \text{ kg m}^{-3}$  (Davis 1994, Ashraf et al. 2006). *A. ampicaps* is used as a source of firewood, it is a good fuel and burns well, and can be converted to charcoal. Banik and Bhosale (1999) report values of 17.24 and  $18.03 \text{ MJ kg}^{-1}$  for the main stem and branches of 3.5-year-old trees. Farmers in northeastern Thailand also use the lower branches for firewood.

Farmers located in northeastern Thailand (near Khon Kaen and Khorat) have established *A. ampicaps* in saline areas as a “live” fence around their houses, and seed and leaves can be fed to chickens, pigs and fish, and young leaves (phyllodes) used as livestock feed.

### 43.5 SOCIAL ACCEPTANCE

Although *A. ampicaps* has been tested in many countries over the last two decades and promoted through government initiatives as a suitable candidate for growing on salt-affected lands as a source of firewood, fodder/forage, agroforestry, and reclamation, it has not gained widespread acceptance. However, the species is making steady inroads into public and private lands.

Siddiqui et al. (2005) conducted a survey to ascertain farmer preferences for *A. ampicaps* vs. *Leptochloa fusca* as a source of fodder in southern Pakistan. The majority of (small) landholders preferred kallar grass, even though *A. ampicaps* was found to be more tolerant to salinity. The main reason for hesitation was lack of awareness about the forage value of this species. The authors conclude that creating awareness of the potential benefits of *A. ampicaps* as well as the role of key farmers may well result in further interest. This work is being conducted as part of the Saline Agriculture Farmer Participatory Development Project in four provinces of Pakistan and *A. ampicaps* is one of several tree species being provided to farmers for assessment.

In northeastern Thailand, *A. ampicaps* acts as the pioneer tree on severely saline soil to lower water table, reduce salinity, and provide a source of fuel wood. It has been promoted as a species for planting in saline areas, including on rice bunds, by government agencies since the mid 1990s (Awan et al. 2007, Yuvaniyama et al. 2008). Between 1996 and 1999, the Government of Thailand implemented a plan for revegetation in both recharge and discharge areas to control secondary salinization in Kham Tale So district, Nakhon Ratchasima province. *A. ampicaps* and *Sporobolus virginicus* (halophytic grass) were planted in severely salinized areas and about 2400 ha of discharge areas were established (Yuvaniyama and Dissataporn 2003). Since 1999, the Land Development Regional Offices have implemented revegetation with *A. ampicaps* along rice paddy bunds on saline land, coupled with surface water management including drainage for 5000 ha of land in Nakhon Ratchasima and Khon Kaen provinces. Since 2009, the Master Plan for Soil Salinity

Integrated Management Projects has been promoted in these provinces to cover 440 ha, including both recharge and discharge areas. *A. ampliceps* is recommended for planting on slightly to moderately saline rice paddy bunds, in blocks, and as scattered trees in higher salinity soils.

### 43.6 CONCLUSIONS

*A. ampliceps*, which occurs naturally in north western Australia, has proven to be well adapted to lighter-textured, moderately high saline and sodic soils, though its growth is markedly restricted by the presence of hard pans and under shallow water table and waterlogging conditions. It has also grown well on sandy, drought-prone soils as long as sufficient water is supplied. These attributes make it a suitable candidate for utilization of degraded lands and their physical, chemical, and biological improvement. Over the last two decades, *A. ampliceps* has been successfully introduced to Pakistan, Thailand, the UAE, and many other countries in Asia/Middle East. Subsequently, the species is making steady inroads into public and private lands. It has the potential to be managed for multiple uses (e.g., fodder, fuelwood, poles, timber) in mixed agricultural systems and to improve salt-affected land.

Establishment of seed orchards of the best performing provenances is necessary for sustained supply of genetically superior seed. There is also a need to select for single stem plants as opposed to many stems from the ground level in most populations. The consequences for salt accumulation in the root zone of *A. ampliceps* needs continued assessment before recommending large-scale planting. The economic benefit of various uses of *A. ampliceps*, especially as animal fodder/forage and timber, needs to be better assessed.

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# 44 Adaptive Strategies of Tropical Forage Grasses to Low Phosphorus Stress: The Case of *Brachiaria* Grasses

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## 44.1 INTRODUCTION

Sustainable development through improved agriculture is vital for meeting the challenges of hunger, poverty, inequality, and environmental degradation in the developing world. Vance et al. (2003) argued that it might be possible to feed the growing world population, but at an accelerated impact on sustainability and environmental quality. In many developing countries, poor soil fertility, improper nutrient management, and a lack of plant genotypes having high tolerance to nutrient deficiencies or toxicities are major constraints contributing to food insecurity, malnutrition, and ecosystem degradation (Cakmak, 2002). Lynch (2007) stated that the most direct contribution to food security would simply be improved food production in developing countries as it directly improves the food security of subsistence farmers, reduces the cost of food for poor consumers, and increases rural incomes. It was suggested that among the three main components of integrated nutrient management that include fertility inputs, soil management and adapted germplasm, adapted germplasm has the leading edge for resource-poor farmers for improving agricultural productivity. Recently, Frossard et al. (2009) emphasized that a package of solutions are needed for the management of agroecosystems and that the benefits from improved germplasm are much greater if strategic phosphorus (P) applications are included. While highly soluble phosphatic fertilizers such as superphosphate and diammonium phosphate are the common sources of P used in arable cropping worldwide, increasing interest exist to use cheaper alternative P fertilizers such as rock phosphates in tropical soils (Lopes et al., 1991; Fardeau and Zapata, 2002). Their slow-release characteristics make them well suited for use in less intensive agricultural systems such as permanent pastures (Haynes, 1992).

*Brachiaria* grasses are the most widely planted tropical forage grasses in the world, covering about 80 million hectares in Brazil alone (Miles et al., 2004; Macedo, 2005; Do Valle and Pagliarini, 2009). Their economic importance for the livestock revolution is greatest in tropical America, where extensive adoption has had a revolutionary impact on the productivity (up to 10-fold increase of animal production per unit area) of vast areas of previously underused, marginal soils (Lascano, 1991). Holmann et al. (2004) studied the adoption of improved *Brachiaria* grasses from 1990 to 2003 in several Central American countries, including Mexico, and estimated the value of the additional production of milk and beef due to the adoption of *Brachiaria* grasses to be around US\$1 billion. These figures support the statement by Delgado et al. (1999) that the structural shift in developing country diets toward animal proteins is a given fact that must be dealt with. This report that summarizes “A 2020 Vision for Food, Agriculture, and the Environment” suggests that livestock production offers one of the few rapidly growing markets that poor, rural people can join even

if they lack substantial amounts of land, training, and capital. Livestock are central to the livelihood of the rural poor in developing countries in at least six ways:

- Livestock is an important source of cash income.
- Livestock is one of the few assets available to the poor, especially poor women.
- Livestock manure and draft power are vital to the preservation of soil fertility and the sustainable intensification of farming systems in many developing areas facing increasing population density.
- Livestock allow the poor to exploit common property resources, such as open grazing areas, in order to earn income.
- Livestock products enable farmers to diversify incomes, helping to reduce income variability, especially in semiarid systems characterized by one cropping season per year.
- Livestock provide a vital and often the only source of income for the poorest and most marginal of the rural poor, such as pastoralists, sharecroppers, and widows.

Rao (2001) considered the selection or breeding of tropical forages adapted to low-fertility acid soils as the most viable approach for increasing pasture and livestock productivity that could contribute to food security in the tropics. Strong support for this idea is provided by the increasing importance of beef and milk in the diet of all economic strata in the tropics and, also, the use of lime to improve soil chemical properties is not economically feasible because the returns in terms of forage yield and quality are low (Miles et al., 2004).

Crop genotypes with greater phosphorus-acquisition efficiency are important contributors to food security in developing countries (Rao, 2001; Lynch, 2007). Jain et al. (2007) explained the importance of sustaining efforts directed toward selection and genetic improvement of P-efficient genotypes. Species that are efficient at accessing sparingly available P could have a favorable effect on other plants used for intercropping or rotation since P-efficient plants could be a viable alternative to the problems of low P availability and excessive P fertilizer use (Ae et al., 1990; Rao et al., 1999b).

Rao et al. (1998) reported that wide genetic diversity in plant attributes for tolerance to infertile acid soils exists in *Brachiaria* germplasm and genetic recombinants and emphasized that this diversity will be utilized in breeding programs to develop superior *Brachiaria* genotypes for infertile acid soils of the tropics. This chapter focuses on experimental approaches and findings obtained by addressing the overall question whether root-based mechanisms, i.e., differences in root growth and root function, could serve as important strategies to enhance P acquisition and might explain differences in soil adaptation between two *Brachiaria* forage grasses, i.e., well-adapted signalgrass (*Brachiaria decumbens* cv. Busilisk) and poorly-adapted ruzigrass (*Brachiaria ruziziensis* cv. Kennedy). Both are used as parents in the *Brachiaria* breeding program (Miles et al., 2004). Our results highlight the importance of analyzing morpho-physiological and biochemical trait profiles and to determine the role of plant phenotypic plasticity to characterize differences in low P adaptation between *Brachiaria* genotypes.

## 44.2 NEED FOR IMPROVING ADAPTATION OF *BRACHIARIA* TO LOW PHOSPHORUS (P) SOILS

### 44.2.1 *BRACHIARIA* FORAGE GRASSES ARE SUITABLE FOR EXTENSIVE AND INTENSIVE AGRICULTURE

*Brachiaria* (Trin.) Griseb. is a large genus comprising more than 90 species from Africa, Asia, Australia, and North/South America. The commercially exploited *Brachiaria* grasses belong to four African species: *Brachiaria decumbens* Stapf (signalgrass), *B. brizantha* (A. Rich.) Stapf (palisadegrass), *B. humidicola* (Rendle) Schweick (koroniviagrass), and *B. ruziziensis* Germain & Evrard

(ruzigrass). Signalgrass, palisadegrass, and ruzigrass are closely related while koroniviagrass falls into a separate group. These few available cultivars play a major role in world livestock production. While the *Brachiaria* forage grasses have been exploited by African pastoralists for millennia, serious interest in the species of *Brachiaria* as sown and managed forage only began in the 1960s. From coastal and humid Australia, the grasses were introduced into tropical South America, beginning in Brazil in the early 1970s and the excellent adaptation of *Brachiaria* grasses to low fertility soils has encouraged their use for extensive, permanent and low-input pastures, but also in more intensively managed pastures. Although the rotation of annual cropping with grazed pasture is not commonly practiced, it is an option for farmers in tropical America following the example of Brazil (Miles et al., 2004).

#### **44.2.2 SIGNALGRASS IS WELL ADAPTED AND RUZIGRASS POORLY ADAPTED TO LOW-FERTILITY ACID SOILS OF TROPICAL AMERICA**

Signalgrass and ruzigrass have similar geographic distributions within only a few degrees of latitude of the Equator in eastern Africa. Both species are from sub-humid to humid environments with a relative short dry season, less than 5 months (Miles et al., 2004). *Brachiaria decumbens* cultivar (cv.) Basilisk (and also CIAT accession 606) derives from seed introduced into Australia from the Ugandan Department of Agriculture in 1930. This cultivar is well adapted to infertile acid soils and forms an aggressive high-yielding sward that withstands heavy grazing and trampling (Keller-Grein et al., 1996).

*Brachiaria ruziziensis* is native to the Ruzi valley in Zaire and Burundi and is widely distributed in tropical countries. The material grown in Australia (and possibly in tropical America) originated from seed received from the Agronomy Station of Lac Aloatra in Madagascar in 1961 and was released under the common name ruzigrass, or also known as cv. Kennedy, which is also the only known cultivar of ruzigrass. Ruzigrass requires fertile, well-drained soils (Keller-Grein et al., 1996). The adoption of cv. Kennedy has occurred in several countries in Southeast Asia, particularly in Thailand, but it is hardly used in tropical America as it is less productive than other cultivars and not well adapted to low-fertility acid soils (Miles et al., 2004).

Miles et al. (2004) also noted that the success of signalgrass on highly acid, infertile soils would not have been predicted, as signalgrass has not been collected from sites with a soil pH below 4.9 while the inability of other forage grasses to compete with *B. decumbens* cv. Basilisk might be attributed to their domestication and selection under conditions of higher soil fertility in Africa or Australia.

#### **44.2.3 BREEDING OF IMPROVED *BRACHIARIA* GRASSES DEPENDS ON OPTIMIZED TRAIT-BASED SELECTION**

The rapid establishment of *Brachiaria* grasses did not occur without problems and available cultivars are now recognized as having serious defects such as sensitivity to spittlebugs, poor edaphic adaptation and inadequate seed filling. No single cultivar combines all the desirable attributes (Rao, 2001; Miles et al., 2004). In addition, millions of hectares of degraded pastures are the result of sowing vast areas of tropical South American savannas to signalgrass and palisadegrass with inadequate fertilization and management. Renovation and intensification of pastures demand new cultivars that are more productive and of better quality even if they require more inputs. Long-term persistence will be less important and soil improvement will be more important in intensive systems of annual crop-pasture rotation (Miles et al., 2004).

Exploiting the natural variability of forage germplasm to identify tropical grass species adapted to the various ecosystems in acid-soil regions has been an important research strategy of CIAT (International Centre for Tropical Agriculture in Cali, Colombia). Breeding efforts started in the mid-1980s to recombine edaphic adaptation found in *B. decumbens* cv. Basilisk with resistance to

spittlebugs, found in *B. brizantha* cv. Marandú. Both species are natural tetraploid apomicts, but produce fertile pollen that can be used to pollinate sexually reproducing tetraploidized *B. ruziziensis* (Miles et al., 2004). Hence, combining genes of the two apomictic species is now possible and hybrids can be further recombined to achieve the desired trait combinations (Rao et al., 1998; Ishitani et al., 2004).

Adapted and promising ecotypes that passed the screening for tolerance to soil constraints, diseases, and insects are further evaluated in terms of tolerance to grazing, minimum nutrient requirements, nutritive value, dry-season performance, compatibility in grass-legume mixtures and economic suitability to fit a particular farming system (Rao, 2001). By the standards of C4 grasses, the nutritive value of the existing *Brachiaria* grass cultivars is good (Miles et al., 2004). Lascano and Euclides (1996) reported that although digestibility of *Brachiaria* grasses is high, variation exists among and within *Brachiaria* species due to different levels of crude protein. Thus, animal performance differs from one *Brachiaria* grass to another. Signalgrass is a palatable grass and gives good animal performance while ruzigrass provides palatable forage of high nutritional quality (Do Valle et al., 1988; Keller-Grein et al., 1996). Betancourt (2006) found that selecting grasses adapted to low-fertility acid soils will not affect forage quality and animal production as the variance associated with genotype x environment interactions was lower than the variance in digestibility.

However, continued progress in the selection and improvement of *Brachiaria* genotypes depends on identifying plant attributes that contribute to tolerance of low-fertility acid soils and the development of rapid and reliable screening methods. An essential part of germplasm selection and improvement is to identify morphological, physiological, and biochemical mechanisms by which forage plants adapt to low-fertility acid-soil conditions (Rao, 2001).

#### 44.2.4 TROPICAL ACID SOILS ARE A RESOURCE FOR FUTURE AGRICULTURAL DEVELOPMENT

Acid soils are found throughout the world, with the largest areas in tropical developing countries and cultivated by poor farmers. The humid tropics accounts for approximately 60% of the acid soils of the world. About 18% of the world's acid soils are used for pastures. In South America, Ultisols and Oxisols contribute 11% and 7%, respectively, to the global figure and to 70% of the P-deficient soils in the tropics. Both Ultisols and Oxisols occur on older land surfaces of the tropics while Oxisols occur mostly near the equator in South America and Africa. Historically, acid soils have resisted permanent settlement and agricultural use. However, this is changing and presently, about 11 million hectares of tropical forest is cleared annually and only a very small portion is converted into highly productive agriculture, whereas most of the remainder becomes less productive grassland. Acid tropical soils do not only represent the most exploited soil resource in the world, but these soils represent a high potential for future agricultural development as there are few climatic constraints to high yield, provided that management is good. The annual production value of the world's acid soils has been estimated to be greater than US\$ 700 billion and for permanent pastures, US\$ 105 billion based on an average production value of US\$ 150 per hectare (Von Uexküll and Mutert, 1995).

#### 44.2.5 LOW P AVAILABILITY IN TROPICAL SOILS IS A LIMITING FACTOR

Poor plant growth in acid soils, i.e., soils with a pH below 5.5 (measured in water) in their surface zones, can be correlated directly with aluminum (Al) saturation. Except for extreme cases, pH has rarely a direct effect on plant growth and H<sup>+</sup> (proton) concentrations may present a constraint only at a very low pH of 4.2. The poor fertility of acid soils is due to a combination of toxicities of Al, manganese, iron (Fe) and deficiencies of P, calcium (Ca), magnesium (Mg), potassium (K), and of low organic matter content (Von Uexküll and Mutert, 1995).

Phosphorus is an essential component of photosynthesis, energy storage, and carbon (C) metabolism, and a general consequence of P deficiency is a decrease in the energy charge of cells (Jain et al., 2007).

A decade ago, Lynch (1998) reported on the central importance of P to agricultural productivity and sustainability in both developing and high-income economies while Abelson (1999) and Cordell et al. (2009) warned that a potential P crisis looms for agriculture in the twenty-first century. Stewart et al. (2005) also reported that low-cost reserves of rock phosphate and much of the known higher-cost reserves will be depleted by 2100.

Frossard et al. (2009) emphasized the importance of analyzing the P input–output balance in intensive and extensive agriculture of the developed and developing worlds, respectively. In intensive agriculture, where P fertilizers are added in excess to plant needs, 20% or less of applied P is removed during the first year of growth because of soil-P retention, and losses from P-loaded soils is a primary factor of eutrophication and hypoxia of lakes and marine estuaries. An even greater concern is the P mining from agricultural soils in the tropics and subtropics where the majority of Earth's people live.

Phosphorus deficiency is a widespread nutritional problem affecting crop production (Cakmak, 2002); and P is second only to nitrogen (N) as the most limiting element for plant growth (Schachtman et al., 1998; Vance et al., 2003). Rao (2001) reported that P is often the most limiting nutrient for pasture establishment and production in highly weathered acid soils of tropical America. Jain et al. (2007) reported an estimate of 5.7 billion ha of land, equivalent to about 67% of the total farmland used worldwide, that contains too low levels of plant available P for sustaining optimal crop production. Soil phosphate ( $P_i$ ) concentrations are typically 60–600 times lower than the concentrations of other macronutrients such as K and Mg (Bieleski, 1973). In many soils,  $P_i$  concentrations in the soil solution range between 0.1 and 10  $\mu\text{M}$  (Frossard et al., 2000) while adequate concentrations required for optimal growth can reach up to tens of  $\mu\text{M}$  for demanding crop species (Ticconi and Abel, 2004). For grasses, an external P requirement between 1 and 5  $\mu\text{M}$  was reported (Hinsinger, 2001). Intracellular plant P concentrations may be 1,000-fold higher than external concentrations (Schachtman et al., 1998).

Highly weathered tropical soils used as grasslands in Latin America are characterized not only by low levels of total and available P, but also by high P-sorption capacity, which is generally associated with type and percentage of clay components (Sanchez and Salinas, 1981; Dubeux et al., 2007). Phosphate is strongly sorbed on iron and aluminum oxides present in highly weathered Oxisols and Ultisols resulting in very low soil-solution  $P_i$  concentrations (Hinsinger, 2001; Bühler et al., 2003; George et al., 2006). Mass flow in soil contributes only up to 5% P to crop uptake while the slow diffusion rate of  $P_i$  and high plant uptake rates result in the creation of a zone around the root that is depleted of P; and the steep concentration gradient in the rhizosphere contributes to favor the diffusion of P (Hinsinger, 2001). An additional factor that limits soil P availability to plants is that plants have to compete with microorganisms for the available P (Oberson et al., 2001; Bünenmann et al., 2004; Jain et al., 2007).

In acid soils,  $P_i$  desorption can be accelerated by the presence of organic acids as these can occupy the sorption sites of  $P_i$  on metal oxides, e.g., Al and Fe oxides (Frossard et al., 1995). This might be relevant for plant nutrition as carboxylates exuded by roots of P-deficient plants may shift the adsorption–desorption equilibrium toward enhanced desorption (Hinsinger, 2001). According to stability constants of complexes with Fe, Al, and Ca, citrate (tricarboxylate) and oxalate (dicarboxylate) are among the most effective compounds with regard to P mobilization. However, in P-deficient soils, significant desorption of  $P_i$  requires usually large amounts of carboxylates in the rhizosphere (Neumann and Römhelt, 2007). Root mucilage, which comprise mainly of polysaccharides and polyuronic acids, can also influence P desorption (Gaume et al., 2000). Evidence exist that certain plant species do not only deplete the most mobile fractions of soil inorganic  $P_i$  (as roots induce a sink effect), but also use the least mobile fractions of soil  $P_i$  (Braun and Helmke, 1995).

Depending on soil type, the organic P content may constitute 20%–80% of the total P present and these organic P forms comprise mainly of phytate, phospholipids, and nucleic acids, which comprise, respectively, up to 50% and between 1% and 5%, of the total organic P. Organic P forms are largely found in the bulk soil, but also in soil solution, often at concentrations greater than

soil solution  $P_i$  and accumulate due to the immobilization of fertilizer P in soil organisms and the build-up of plant residues that are resistant to mineralization (Randall et al., 2001). George et al. (2006) reported that P cycling from organic pools, rather than equilibration of the soil solution with bound inorganic P, is important in P-deficient Oxisols and Ultisols. Extracellular acid phosphatases (APases) with broad substrate specificity released by plant roots may play a role in obtaining  $P_i$  from soil organic P compounds (Vance et al., 2003).

Plant uptake of P is facilitated by low- and high-affinity plant uptake systems operating, respectively, at high and low plant P concentrations while processes such as conversion of  $P_i$  into organic storage compounds, decreases in P uptake rates, and efflux of  $P_i$  contribute to plant- $P_i$  homeostasis (Schachtman et al., 1998; Vance et al., 2003). Alternatively, plants can establish symbiotic associations with mycorrhizal fungi and mycorrhizal plants can acquire  $P_i$  either directly from the soil through plant-specific transporters or through uptake and transport systems of the fungal symbiont (Martin et al., 2007).

#### 44.2.6 SELECTION OF P EFFICIENT *BRACHIARIA* GRASSES

Rao (2001) suggested that plant attributes of *Brachiaria* grasses conferring adaptation to low-fertility acid soils appear to be linked to different strategies to acquire and use nutrients. Scientific support also exists that screening for components responsible for differences in P acquisition or utilization of P by *Brachiaria* genotypes appears to be viable.

Shoot and root growth of *Brachiaria* grasses is responsive to P fertilization and yield increases following P applications have been reported in field (Rao et al., 1998) and in pot experiments (Rao et al., 1996a, 1999a). More specifically, elucidation of the physiological basis of acid-soil adaptation of *Brachiaria* grasses indicated that the high level of adaptation of apomictic *B. decumbens* (cv. Basilisk) is due to its superior resistance to toxic levels of Al (Wenzl et al., 2001), combined with excellent adaptation to P and N deficiencies (Rao et al., 1996b). Rao et al. (1998) subjected 55 *Brachiaria* genotypes to field evaluation, including signalgrass (CIAT accession 606) and a tetraploid sexual ruzigrass that facilitated *Brachiaria* breeding. Ruzigrass was the least efficient genotype in acquiring P and N and also, the least persistent in the short term (i.e., 5.5 months after pasture establishment) when various plant parameters, including biomass production, were compared. Field studies also indicated that diploid sexual ruzigrass is better than tetraploid sexual ruzigrass during the first 6 months of pasture establishment, but even the diploid ruzigrass does not persist beyond 2 years in low-P acid soils (CIAT, 1995, 2007; Rao et al., 1998; Ricaurte et al., 2007). In addition, the enhanced secretion of phytases, a subtype of APases that hydrolyzes P from phytate, by signalgrass during P limitation was reported by Li et al. (1997).

### 44.3 MORPHO-PHYSIOLOGICAL MECHANISMS OF LOW-P ADAPTATION

#### 44.3.1 SOLUTION CULTURE SYSTEM TO STUDY PLANT ADAPTIVE RESPONSES TO LOW P SUPPLY

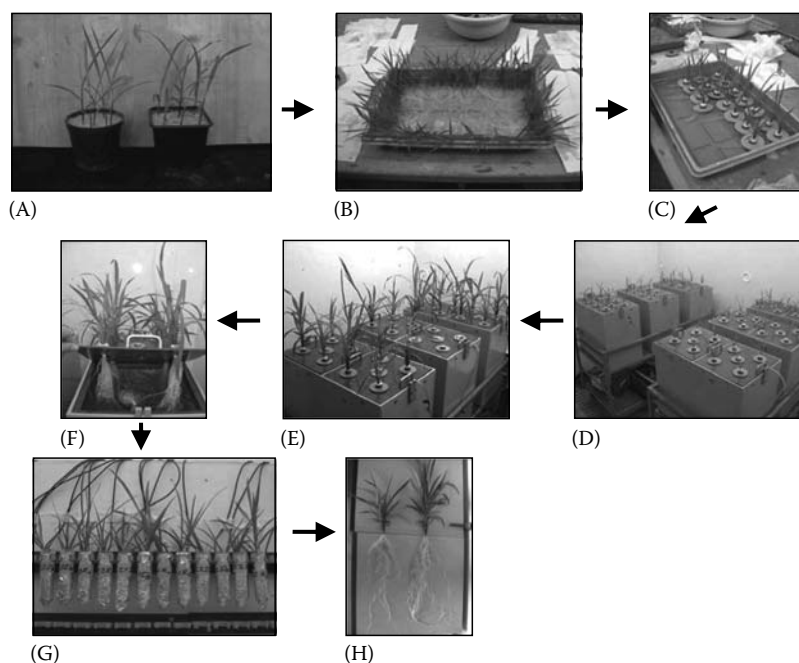
Although the usefulness of natural or field conditions cannot be over-emphasized, it is often advisable to conduct studies aimed at genotype selection under controlled conditions to avoid the confounding effects of the environment and management. Several workers in screening trials have opted for soil-less media such as the nutrient solution culture technique, which does not only save time, labor, and space, but plants could be exposed to adequate nutrition and be free from interferences of soil colloids. Studies of root growth and function are especially easier in nutrient solution (Ahmad et al., 2001).

To approximate P supply in nutrient solutions, media with a constant low P supply is recommended, e.g., the use of either rock-phosphate or tri-calcium phosphate as an insoluble P source will not only buffer P supply in the solution, but will also provide a uniformly dispersed adsorbing material (i.e., Ca) to more closely approximate factors critical for P availability in soils (Ahmad et al., 2001). Sas et al. (2001) demonstrated the suitability of hydroxyapatite as a P source for

*Lupinus albus* grown in nutrient solution. This system uses hydroxyapatite suspended in a dialysis pouch and Pi diffuses out of the pouch as a function of pH. The suitability of this system was tested and implemented for morpho-physiological and biochemical studies of signalgrass and ruzigrass at different levels of P supply (Louw-Gaume et al., 2010).

Figure 44.1 outlines the main steps for the growth of signalgrass and ruzigrass using the hydroxyapatite pouch system in hydroponics at pH 5.5. For the low P treatment, it was possible to reach concentrations of  $1\ \mu\text{M}$ , which is in agreement with the value used by Wenzl et al. (2003) to stimulate soil solutions of highly weathered acid soils. The adoption of the hydroxyapatite pouch system was necessary to obtain a better estimate on the distribution of Pi in nutrient solution over time and since we focused on temporal differences in plant responses during the development of plant-P deficiency. The design of this nutrient-based experimental setup, which was also used to grow plants with the purpose to collect root exudates, considered the recommendations by Neumann and Römheld (2000).

One of our goals was to obtain physiologically relevant data and this is because Jones et al. (2004) cautioned that most studies demonstrating a link between plant nutrient status and markers of P deficiency, such as root exudation components, report results where a general breakdown in plant metabolism occurred. The precise timing of P deficiency-induced gene expression is also unknown due to the use of different experimental techniques to impose P deficiency (Hammond et al., 2004). Plant developmental stage can also affect root exudation of carboxylates (Neumann and



**FIGURE 44.1** A scheme showing steps involved in the growth of *Brachiaria* grasses in hydroponic conditions at pH 5.5 using the hydroxyapatite-containing dialysis pouch system for the slow release of Pi as function of pH (pH was titrated on a daily basis using KOH solution). The evolution of Pi was monitored on a daily basis. Nutrient solution and dialysis pouches were changed on weekly basis. (A) Pre-grown seedlings (10–12 days old) in sand culture and grown with minimal fertilization. (B) Washed seedlings ready for selection. (C) Preparation of uniform seedling bunches (three plants per bunch). (D) Transfer of seedling bunches to hydroponic containers containing apatite-containing dialysis pouches (P supply source) and all macro/micro nutrients. (E) *Brachiaria* plants grown in nutrient solution with Pi released from apatite source. (F) Hydroxyapatite-containing dialysis bag shown at bottom of figure. (G) Collection of root exudates in  $\text{CaCl}_2$ -solutions with proteinase inhibitor (shown for young plants). (H) Visible differences in biomass production between signalgrass (on the left) and ruzigrass (on the right).



Römheld, 2007) and moreover, the buffering capacity of cytoplasmic Pi resulting from vacuolar stores contributes to the complexity of resolving the sequence of physiological effects of P deficiency (Sinclair and Vadez, 2002; Ticconi and Abel, 2004).

Hammond et al. (2004) suggested to group changes in Pi-responsive gene expression into early genes that respond rapidly and often nonspecifically, and later responses that alter the morphology, physiology, or metabolism upon prolonged P deficiency. Rao (2001) noted that the late responses might be the ecologically relevant ones. Our investigations focused on the later responses, i.e., from day 7 (young plants) up to day 30 (adult plants) after inducing low P treatments as growth experiments for periods longer than 5 weeks are complicated by flowering, which appears not to be synchronized within and between the two grasses. When P availability was really low, both grasses maintained their size as young seedlings for a long period and grew very slowly, making it difficult to study plant responses that are relevant to field conditions. Kerguelén et al. (2009) encountered similar problems during phenotypic screening of *Brachiaria* hybrids, including signalgrass and ruzigrass as parental material, and did not find differences in many of the morphological and physiological traits investigated among the two parents during a six-week evaluation in an Oxisol containing low levels of available P and toxic levels of Al.

Preliminary experiments indicated the necessity to pre-grow seedlings in sand culture (see Figure 44.1) to obtain plant material of a decent size to prevent problems of mortality associated with the use of very small seedlings in hydroponic containers of 30 L, a volume that was appropriate for the root systems of adult plants grown at high P supply.

#### **44.3.2 PRE-EXPERIMENTS INDICATED THAT RUZIGRASS IS MORE RESPONSIVE TO P FERTILIZATION**

Although this chapter elaborates on approaches and results obtained for hydroponically grown signalgrass and ruzigrass, pre-experiments testing various growth systems, including pots with soil or sand culture and nutrient solution systems, indicated unforeseen biomass differences between the two grasses. Ruzigrass was more responsive to P fertilization on the short term (up to 35 days) and accumulated biomass faster than signalgrass. This response was evident at very low levels of P supply while the two grasses did not differ in plant P concentrations, reaching levels below 0.1 mg P g<sup>-1</sup> dry matter (DM). Reported shoot P concentrations of *Brachiaria* grasses indicated values below 0.10% where values above 0.12% are considered adequate to meet animal requirements (Rao, 2001). Phosphorus deficiency symptoms occur when plant P concentrations decline below 0.1–0.2 mg P g<sup>-1</sup> DM (Fredeen et al., 1989).

These findings also led to the hypothesis that signalgrass might have attributes as described for wild plants that are adapted to infertile environments while ruzigrass might have morphophysiological and biochemical traits that are more similar to domesticated crop species. In addition to the role of fine root growth and root exudation of carboxylates and APases, growth relations between roots and shoots (including a distinction at leaf and stem level) became an additional focus area, not only in terms of differences in biomass production, but biomass partitioning in response to P stress were also studied. Lambers et al. (2006) highlighted that both morphological and physiological mechanisms contribute to enhance P efficiency in Proteaceae and other endemic species of Australia.

#### **44.3.3 BIOMASS PRODUCTION OF SIGNALGRASS AND RUZIGRASS**

##### **44.3.3.1 Signalgrass Is a Slower-Growing Grass with Higher Shoot Mass Density**

More than 20 years ago, Rorison (1986) reported that plants most capable of surviving on acid soils tend to be those with inherently slow rates of growth, while Helyar (1994) emphasized that plants adapted to nutrient-poor soils are often adapted to survive rather than to be productive. Ryser (1998)

suggested that nutrient-poor habitats will be dominated by slow-growing species with low nutrient-loss rates and long tissue lifespan, and nutrient-rich habitats by fast-growing species with high rates of nutrient loss and short tissue lifespan.

Berendse et al. (1999) suggested that an increase in the length of the time period during which nutrients can be used is an alternative mechanism through which plants can adapt to nutrient-poor soils. The length of this time period can be expanded by increasing the lifespan of leaves, roots, and other organs and longer tissue lifespan improves nutrient conservation and nutrient-use efficiency. Lifespan can be increased by investing in sclerenchymatous and structural tissues and in defense compounds that reduce the risks of herbivory. Vance et al. (2003) also considered sclerophylly, an outcome from enhanced phenolic metabolism and cell wall lignification, as an adaptation by Proteaceae that occupy low-P habitats. Ryser and Urbas (2000) also reported that leaf longevity is more important than nutrient resorption from senescing leaves, while Berendse et al. (1999) reported that nutrient resorption from dying roots is minimal. High plant-tissue mass densities have been associated with slow growers (Poorter and De Jong, 1999; Ryser and Urbas, 2000) and are characterized by high plant-C concentrations and long leaf and root longevities (Ryser, 1998). Schläpfer and Ryser (1996) assumed a tight relationship between tissue volume and fresh mass, and concluded that the tissue dry biomass content, i.e., the tissue DM-to-fresh biomass (FM) ratio might reflect tissue mass density. This relationship was used to express leaf mass density (LMD), stem mass density (SMD), and root mass density (RMD) of signalgrass and ruzigrass (Louw-Gaume et al., 2009).

A comparative study confirmed higher biomass production for ruzigrass, up to 50% more, for low and high P-supply levels while signalgrass was a slower-growing grass with higher LMD and SMD during a growth period of 30 days (Louw-Gaume et al., 2010). A lower growth rate has been suggested to be an effective mechanism to cope with low P soils (Lajtha and Harrison, 1995). The higher capacity for biomass production in diploid sexual ruzigrass on the short-term in hydroponic conditions was not totally unexpected based on field observations where this grass had rapid establishment, but its performance declined over time under low P supply conditions in acid soils. Other short-term studies also confirmed that potentially fast-growers species can outperform potentially slow-growers under nutrient limitation (Chapin, 1980; Poorter et al., 1995) while a simulation study of long-term biomass dynamics of perennials under low N inputs showed that despite higher initial growth rates of fast-growers, species with slower tissue turnover gained advantage over time (Aerts and Van der Peijl, 1993).

The higher shoot mass density and higher shoot C concentrations of signalgrass may contribute to reduced nutrient requirements in the long term (Louw-Gaume, 2009; Louw-Gaume et al., 2009). Nord and Lynch (2008) also argued that because P in photosynthetic processes is recycled, longer leaf lifespan should increase the yield of photosynthate per unit of leaf P. The LMD increased only in low-P grown ruzigrass (Louw-Gaume, 2009) while responsiveness of this phenotypic trait to variation in P supply was reported by Ryser and Eek (2000). An increase in LMD might reflect preferential investment in cell wall compounds instead of cytoplasmic compounds (Poorter et al., 1995) and a switch from primary to secondary metabolism (Vance et al., 2003). Low-P plants of ruzigrass with increased LMD also had a reduced capacity for biomass production, suggesting the development of a no-win situation for ruzigrass due to higher biosynthetic costs and increased competition for C between growth and that required for up-regulated secondary metabolism (Vazquez de Aldana and Berendse, 1997; Heil and Baldwin, 2002; Smith and Stitt, 2007). Young seedlings of ruzigrass also had lower shoot mass densities, which allowed rapid growth, even with minimal nutrient supply, a response that complicated the selection and standardization of seedlings (Louw-Gaume et al., 2009).

Our findings for signalgrass as a slow growing grass, together with the high level of Al resistance as found by Wenzl et al. (2001), support the idea that stress-tolerant grasses with a growth conservative strategy, adapted to acid soils, are more Al resistant (Poozesh et al., 2007).

44.3.3.2 Ruzigrass Decreases Its Growth Rate to Cope with Low P Supply

Plant adaptations directed at the enhancement of P use also include a decrease in growth rate, more growth per unit P taken up and modified C and N metabolism (Lajtha and Harrison, 1995). In contrast with low-P grown signalgrass in which growth rate was not affected by the plant’s physiological P status, ruzigrass reduced its growth rate when the plant’s P concentrations declined from 0.2 mg to 0.1 mg P g<sup>-1</sup> DM during a three week growth period including harvests at day 7, day 14, and day 21 (Louw-Gaume, 2009). This experiment investigated temporal patterns of plant growth and root physiology during adaptation to low P supply and species responses and differences are summarized in Table 44.1.

Simultaneously, leaf expansion (measured as the increase in leaf area production) was reduced while root elongation (determined as the increase in root length production) was stimulated as compensating morphological adaptations to cope with limiting availability of P for growth. Various studies showed that declining P availability influences leaf and root growth, e.g., root development at low P supply has been sustained or even stimulated (Rufty et al., 1993; Mollier and Pellerin, 1999) while reduced leaf expansion was the first response to decreased P supply in soybean (Fredeen et al., 1989) and in sugar beet (Rao and Terry, 1989). Mollier and Pellerin (1999) reported that leaf area development in maize decreased long before radiation-use efficiency. Sinclair and Vadez (2002) noted that photosynthetic rates are stable over a wide range of decreasing P levels, but developing cells of P-deficient plants lack mature vacuoles and a large reserve of P to buffer growth, and are especially vulnerable to low P supply. Rao et al. (1996a, 1999a) reported that shoot and root growth

**TABLE 44.1**  
**Temporal Differences in Morphological and Physiological Traits**  
**of Signalgrass and Ruzigrass at Three Harvest Intervals: Day 7 (D7),**  
**Day 14 (D14), and Day 21 (D21)**

| Day 7                                              | Day 14                                                      | Day 21                                                                                                                                                                 |
|----------------------------------------------------|-------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Ruzigrass</i>                                   |                                                             |                                                                                                                                                                        |
| Higher growth rate<br>(more biomass<br>production) | Higher growth rate<br>Oxalate exudation<br>sAPase secretion | Growth rate similar to signalgrass (<D14)<br>Oxalic acid exudation rate similar to signalgrass (=D14)<br>Lower oxalate-to-lactate ratio                                |
| Higher plant-P<br>concentrations                   | Root carboxylate<br>concentrations<br>variable              | sAPase secretion similar to signalgrass (=D14)<br>Higher cell-wall-associated APase secretion<br>Higher root-to-shoot ratio<br>Lower root carboxylate concentrations   |
| <i>Signalgrass</i>                                 |                                                             |                                                                                                                                                                        |
| Higher shoot mass<br>density                       | Root carboxylate<br>concentrations                          | Growth rate = D7 (and D14)<br>Higher shoot mass density = D7 (and D14)                                                                                                 |
| Higher plant C<br>concentrations                   | maintained                                                  | Plant C concentrations = D7 (and D14)<br>Stem mass fraction = D7 (and D14)                                                                                             |
| Higher stem mass<br>fraction                       |                                                             | Oxalic acid exudation<br>Oxalate dominant exuded carboxylate<br>sAPase secretion<br>Cell-wall-associated APase secretion<br>Root carboxylate concentrations maintained |

*Note:* The two species are compared, e.g., plant growth rate decreased in ruzigrass between day 14 and day 21 to a similar rate as in signalgrass while growth rate was unaltered for signalgrass. sAPase: secreted acid phosphatases. Experimental conditions are described by Louw-Gaume (2009). Decreasing plant P concentrations reaching 0.1 mg P g<sup>-1</sup> DM at D21 in both grasses.

**TABLE 44.2**  
**Leaf Length and Leaf Width of Signalgrass**  
**and Ruzigrass Grown at Low and High**  
**P Supply**

| P Treatment           | Signalgrass       |                   | Ruzigrass         |                   |
|-----------------------|-------------------|-------------------|-------------------|-------------------|
|                       | Leaf Length (cm)  | Leaf Width (cm)   | Leaf Length (cm)  | Leaf Width (cm)   |
| Low P                 | 23.3 <sup>a</sup> | 1.30 <sup>a</sup> | 31.8 <sup>b</sup> | 1.50 <sup>b</sup> |
| High P                | 32.6 <sup>b</sup> | 1.56 <sup>b</sup> | 41.5 <sup>c</sup> | 1.78 <sup>c</sup> |
| % Low P versus high P | 72                | 83                | 77                | 84                |

*Note:* Small letters indicate significant differences between grasses and P treatments. Experimental conditions are described by Louw-Gaume (2009).

of *Brachiaria* grasses are responsive to variation in P supply. Our results confirm these observations and comparisons of shoot and root growth showed that shoot growth was prioritized at high P supply and root growth at low P supply in both grasses (Louw-Gaume et al., 2010).

A comparison of leaf length and leaf width (5 leaves per plant; a total of 24 plants of each grass) showed similar values for leaf length and width in signalgrass grown at high P supply and for ruzigrass at low P supply (Table 44.2). Leaf-growth parameters also differed between the two grasses, e.g., leaf length at low P relative to that of high P (expressed as %) was lower than the leaf width comparison, and the difference was more evident for signalgrass. Kavanová et al. (2006) noted that P deficiency decreases leaf elongation of grasses.

**44.3.4 BIOMASS ALLOCATION PATTERNS OF SIGNALGRASS AND RUZIGRASS**

**44.3.4.1 Tissue Mass Fractions and Root-to-Shoot (R:S) Ratios**

The importance of biomass allocation for yield improvement has been highlighted a long time ago (Gifford et al., 1984). Rao (2001) reported that the adaptation of forage plants to acid soils also involved changes in the partitioning of biomass between shoots and roots. Not only is nutrient deprivation a controlling factor of biomass production and yield (Marschner, 1986), but biomass allocation patterns are also influenced by nutrient levels. Biomass allocation to roots is an important determinant for nutrient acquisition: first, the bulk of the nutrients enter the plant by roots and/or mycorrhizal uptake and second, it is well documented that plants allocate relatively less biomass to leaves and more to their roots when N or P are in short supply (Poorter and Nagel, 2000), particularly in fast-growing species (Aerts and Chapin, 2000).

According to Atkinson (2000), the root-to-shoot DM ratio (R:S ratio) serves as a benchmark of the plant nutritional status while Poorter and Nagel (2000) suggested to distinguish among leaf, stem and root mass fractions (LMF, SMF, and RMF, respectively) as these organs have very different functions. Both approaches were included and tissue mass fractions of the two grasses were calculated as the ratio of DM of a particular organ to the total plant DM. Low-P grown plants of both grasses had higher R:S ratios and RMF than high-P grown plants (Louw-Gaume et al., 2010).

At low P and high P supply, the SMF was greater for signalgrass (Louw-Gaume et al., 2010), a significant result for understanding its superior adaptation to low-P acid soils as higher allocation of biomass to stems was reported for highly productive grasslands compared with less productive

grasslands (Poorter and De Jong, 1999). Signalgrass is described as a trailing perennial with up to 60 cm stems and was named after its decumbent growth habit (FAO, 2008). Gibberellins (GAs) strongly promote growth of stems and, to a lesser extent, that of leaves and roots (Nagel and Lambers, 2002). In this study, low-GA mutants of tomato had reduced growth rates and allocate a smaller fraction of C to leaf growth and a much larger fraction to root respiration. Findings from this study also led to the suggestion that GAs might have a direct role in explaining variation in relative growth rate (RGR), which is an alternative view to that where RGR is casually linked to variation in rates of photosynthesis and respiration. Investigations into GAs might contribute to unravel the underlying mechanisms of slower growth of signalgrass and the reduced growth rate in P-deficient ruzigrass. Jiang et al. (2007) found that the effects of P deficiency on root elongation were modulated by DELLA proteins, core components of the GA-signaling pathway. The role of GAs and DELLA proteins on plant growth was recently reviewed by Achard and Genschik (2009).

#### **44.3.4.2 Growth under Low P Supply Increases Biomass Allocation to Roots and Root Carbon Concentrations**

Starch levels have been shown to increase in P-deficient leaves (Fredeen et al., 1989; Rychter and Rao, 2005) while greater R:S ratios under low P conditions have been attributed to enhanced C allocation to roots and greater amounts of carbohydrates in P-deficient roots (Mollier and Pellerin, 1999; Hermans et al., 2006). This might be the outcome when shoot growth is more affected than photosynthesis (Rao and Terry, 1995; Marschner, 1998; Poorter and Nagel, 2000). Although low-P grown adult plants of both grasses did not differ in absolute values for R:S ratios and root C concentrations, both parameters increased when plant P concentrations declined from 0.2 to 0.1 mg P g<sup>-1</sup> DM (Louw-Gaume, 2009; Louw-Gaume et al., 2010). Nutrient-stressed plants may use an excess of relatively unlimited C for the acquisition of the limiting nutrient and therefore, increased root surface area under low P supply conditions suggests that low-P plants may expend energy or invest plant resources for the purpose of acquiring limiting P (Bates and Lynch, 2000).

### **44.3.5 LATERAL ROOT GROWTH AND OTHER ROOT TRAITS OF SIGNALGRASS AND RUZIGRASS**

#### **44.3.5.1 Signalgrass Maintains Lateral Root Growth in Response to Variation in P Supply**

Root growth is decisive for establishing soil/root contact and for the uptake of soil and fertilizer P by crops as P transport is the main limiting factor for P acquisition in most soils (Horst et al., 2001). Rao (2001) reported that root attributes such as length, surface area, fineness and density of root hairs influence plant adaptation to low-P soils. The uptake of P is generally considered to be proportional to the surface area of the plant organs involved in P uptake and thus, a finely divided and rapidly developing root system will provide better access to the less mobile Pi ion (Atkinson, 2000).

We investigated lateral root and main root proportions by cutting the finer laterals from the thicker seminal and main roots, which were collectively designated as the main root fraction. The lateral root fraction, as a percentage of the total DM of the whole root system, was similar for low-P- and high-P grown plants of signalgrass, but for ruzigrass, lateral root growth was stimulated at low P supply (Louw-Gaume et al., 2010). The redesign of root system architecture to accelerate soil exploration is an adaptive plant response associated with the maximization of external Pi acquisition (Ticconi and Abel, 2004). Osmont et al. (2007) highlighted that P deficiency can have variable effects in Arabidopsis as some accessions showed a reduction in the growth of both primary roots and lateral roots while others showed only one of the two or even an inverse response. These effects might be the outcome of reduced meristematic activity and a loss of auxin responsiveness in the root meristem.

Maintenance of root growth and extensive root systems has been suggested to underlie edaphic adaptation of *Brachiaria* genotypes (Rao et al., 1996a,b; Miles et al., 2004). Our results suggest that for signalgrass, growth maintenance of fine lateral roots might be a constitutive (genetic) attribute, a finding fitting the notion that ecological specialization on infertile soils involves the evolution of extensive root systems, which remain functional throughout the year, but with relatively inflexible patterns of root development (Crick and Grime, 1987). Together with the observation that ruzigrass increased lateral root growth at low P supply (Louw-Gaume et al., 2010), it appears that a large root system is either an inherent property or may be nutrient deficiency-induced (Marschner, 1998).

Higher root densities might help signalgrass to explore a large soil volume for P due to the large number of fine roots per unit of soil volume, and one advantage might include the ability to intercept unpredictable nutrients and short-lived nutrient pulses in heterogeneous soil-nutrient environments (Hutchings and De Kroon, 1994; Hodge, 2004). In addition, plants with high root densities have been associated with high agronomic effectiveness of rock phosphates and significant yield responses for signalgrass after the application of rock phosphates (10–13 months) have been reported (Sale and Mokwunye, 1993). Maintenance growth of lateral roots also contributed to a stronger feedback loop on shoot growth at a more optimal level of P supply as evident by a greater leaf-to-root DM ratio in high-P grown plants (Louw-Gaume et al., 2010). McCully (1999) emphasized that in corn, fine roots play an active role in root function and even the oldest fine roots are active in P uptake as they are well supplied with assimilates and have extensive phloem connections. Lynch and Nord (2008) also highlighted that root length duration, i.e., the integral of root length over time from germination to harvest is important for plant P acquisition. Arredondo and Johnson (1999) suggested that the overall growth rate might increase if more C was available for shoot re-growth and if the capacity for soil nutrient acquisition was greater. Furthermore, Berendse et al. (1999) stated that high C costs required for the maintenance of a large viable root mass may not be a problem in nutrient-poor environments as C is not the limiting factor.

#### 44.3.5.2 Root Mass Density Explains Variation in Specific Root Length

Fitter (2002) highlighted that the benefits derivable from a given investment in root DM depend on root system architecture. Specific root length (SRL), calculated as root length per unit root DM, is a root trait that could reflect these benefits as many root functions, such as water and ion uptake, are more closely related to root length than to root volume. High values of SRL can imply a greater ability to obtain such resources and has been found in young root systems and those grown in low-nutrient soils. In agreement, young root systems of low-P grown plants (7 days after inducing low P treatment) had higher SRL than older plants (21 days) in both grasses (Louw-Gaume, 2009).

The reduction in SRL has been attributed to a reduction in root diameter (Fitter, 2002), but SRL is not a simple function of root diameter and may be more closely related to RMD (Ryser, 2006). In our studies, RMD explained most of the variation in SRL and both grasses had lower RMD in younger low-P grown plants than in older plants (Louw-Gaume, 2009). BassiriRad (2005) included root longevity as a rhizospheric trait affecting nutrient uptake in a newer model depicting the major factors of plant nutrient acquisition. Plants vary widely in root lifespan and this has consequences for nutrient acquisition (Wahl and Ryser, 2000; Eissenstat and Volder, 2005). Ryser and Lambers (1995) found that RMD increased in response to low nutrient supply, possibly due to the loss of root cortex layers (Robinson, 1990; Koch, 1996). Low-P and high-P adult plants (30 days after inducing P treatment) of the same grass did not differ in RMD and also, the two grasses did not differ (Louw-Gaume et al., 2010). McCully (1999) reported that older roots of many grasses shed variable amounts of cortical tissue; thus, plant ontogeny is an important feature to include when holistic understanding of root responses to nutrient stress is sought.

Wenzl et al. (2000) found that roots of P-deprived signalgrass and ruzigrass accumulated two di-hydroxycinnamoylquinic acid esters and suggested that these compounds might retard root senescence and act as antifungal compounds. As observed for RMD (Louw-Gaume, 2009), the presence of these hydroxycinnamate conjugates could be associated with enhanced biomass partitioning to roots. A possibility is that these compounds might serve as metabolite markers to follow changes in RMD in *Brachiaria* grasses in response to P stress.

The two grasses did not differ in SRL in an experiment that investigated the role of various mycorrhizal strains on P acquisition and SRL decreased in a similar way for both grasses upon high levels of mycorrhizal root colonization (A. Louw-Gaume unpublished data). Eissenstat and Volder (2005) reported that the response of SRL to environmental conditions cannot always be predicted. Notwithstanding, plasticity of SRL and stability of R:S ratio are important attributes to maintain plant growth rate in infertile habitats as their interplay permits for the maximum amount of DM to be produced for the minimum degree of morphological change (Robinson and Rorison, 1988).

#### 44.3.5.3 Signalgrass Stores Nutrients in Main Roots at More Optimal P Supply

Shoot and root concentrations of not only P, but also of K, Mg, and Ca were investigated in main roots and lateral roots as a function of P supply (Louw-Gaume et al., 2009). Interest in nutrient distribution patterns between shoot and root compartments and within root types was encouraged by various observations:

- Nutrient accumulation is an important ecological strategy of perennials (Chapin, 1980).
- Slower growth contributes to higher nutrient uptake relative to nutrient utilization during periods of higher nutrient availability (Grime, 1977).
- Nutrient storage in thick roots is important in perennial grasses that reallocate nutrients to the roots at the end of the growing season (Boot and Mensink, 1990; Ström et al., 2005).
- Roots play a role in Pi homeostasis as a Pi source during Pi sufficiency and as a sink for Pi during Pi deprivation (Jain et al., 2007).

Higher shoot concentrations of K, Mg, and Ca of ruzigrass at both levels of P supply provided further evidence that this grass is a faster-growing *Brachiaria* species with a higher nutrient demand (Louw-Gaume et al., 2010). Observations for signalgrass support the notion that slow growers are less responsive to increased nutrient availability (Ryser and Lambers, 1995). Rao et al. (1996b) reported that ruzigrass has a higher Ca requirement. For P, only the shoot concentration of ruzigrass was higher at high P supply, supporting findings that low-P grown *Brachiaria* grasses have similar plant P concentrations (Rao, 2001).

Both grasses had lower shoot and root concentrations of P, Mg, and Ca when grown at low P supply, but not for K. Shoots and roots had similar nutrient concentrations in low-P grown plants of both grasses, but at high P supply, root concentrations of signalgrass were higher than for shoots due to a greater increase in root P, Mg, and Ca concentrations (i.e., from 80% to almost threefold). For ruzigrass, shoot and root concentrations increased to a similar extent (70%–100%) at high P supply. Low-P grown signalgrass also had higher root P than shoot P concentrations (Louw-Gaume et al., 2010).

Chapin and Bielecki (1982) reported that root-P retention resulted in higher P concentrations in roots than in shoots in barley while Jain et al. (2007) suggested that root-P retention is an adaptive mechanism to maintain Pi homeostasis at the whole plant level. Comparison of nutrient concentrations between root types showed that lateral roots of low-P grown signalgrass had higher concentrations of P, Mg, K, and Ca. For high-P grown plants, main roots had higher concentrations, suggesting that main roots of high-P grown signalgrass can accumulate nutrients when P availability for growth becomes more optimal. This plasticity in nutrient partitioning between root classes was not evident for ruzigrass (Louw-Gaume et al., 2010).

## 44.4 BIOCHEMICAL MECHANISMS OF LOW P ADAPTATION

### 44.4.1 ROOT EXUDATION PATTERNS OF OXALATE AND ACID PHOSPHATASES AS AFFECTED BY THE PLANT PHYSIOLOGICAL STATUS IN SIGNALGRASS AND RUZIGRASS

Not only do plant roots respond to P deficiency through increased root growth and lateral root formation, but enhanced expression of P transporters and exudation of carboxylates (e.g., citrate, malate, and oxalate) and APases that increase P availability in the rhizosphere are typical responses (Horst et al., 2001; Vance et al., 2003). The exact mechanisms are unclear, but these root exudation components allow plants to increase the pool of soil P, which contributes to plant P nutrition. In fact, Veneklaas et al. (2003) suggested that the key player in plant–soil interactions might be rhizosphere chemistry instead of root morphology.

Temporal release curves of oxalate and secreted-APase by both grasses matched those of decreasing plant P concentrations, suggesting that oxalate and APases were induced and exuded to improve P acquisition from less available inorganic and organic P forms from highly weathered tropical acid soils (Louw-Gaume, 2009). Support that a threshold of Pi depletion during the transition from P sufficiency to P deficiency activates Pi starvation responses such as alterations in root growth and function is provided by

- Findings from *Arabidopsis pho2* mutants that over-accumulate Pi strongly suggest that internal Pi concentrations are important for root morphological changes (Osmont et al., 2007).
- Critical leaf Pi concentrations may trigger carboxylate exudation (Shane and Lambers, 2005).
- Correlations between the intracellular and/or extracellular APase activity and cellular Pi status exist (Duff et al., 1994).

Low-P-induced root exudation of citrate and malate have been shown for white lupin (Ryan et al., 2001; Neumann and Martinoia, 2002; Vance et al., 2003), various Proteaceae species (Shane and Lambers, 2005; Denton et al., 2007) and in maize (Gaume et al., 2001a), but was not detected in our studies with signalgrass and ruzigrass. In addition to oxalate, monocarboxylates such as acetate, glycolate, formate, and lactate comprised the major exuded carboxylates in both grasses. As expected, monocarboxylate exudation could not be related to the plant-P status in both grasses (Louw-Gaume, 2009), but Jones (1998) noted that monocarboxylates could change the kinetics of soil P sorption. Carboxylate exudation might also be a constitutive trait with limited P benefits in some species as found for chickpea (Pearse et al., 2007).

The qualitative differences in carboxylate exudation between low-P grown plants of both grasses (Louw-Gaume, 2009) also opens up the possibility that signalgrass and ruzigrass might be selecting for different beneficial rhizosphere communities through differential root exudation patterns as suggested by various rhizosphere investigators (Marschner et al., 2004; De Boer et al., 2006; Micallef et al., 2009). Interestingly, De Silva et al. (2002) reported that non-sporing gram-positive and gram-negative rods that utilize lactose were favored in soils cultivated with ruzigrass, a response that was influenced by liming and P fertilization. Van Noordwijk et al. (1998) even suggested that if rhizosphere activity helps to alleviate plant nutrient shortages, it may form a negative feedback loop on the nutrient deficiency, similar to the overall shoot-to-root equilibrium.

### 44.4.2 OXALATE EXUDATION MIGHT PRESENT SIGNALGRASS WITH LONG-TERM ECOLOGICAL BENEFITS

Oxalate is a common constituent of plants (Libert and Franceschi, 1987); and a survey of 24 tropical grasses reported values for oxalate contents between 0.02% and 2.5% (Garcia-Rivera and Morris, 1955). Oxalate exudation in response to P limitation has been reported in oxalate accumulators



(Gerke et al., 2000), soybean (Dong et al., 2004; Liao et al., 2006), rice (Hoffland et al., 2006), and *Banksia* species (Denton et al., 2007). Exudation rates of oxalate and secreted-APases in adult plants did not differ between grasses (Louw-Gaume, 2009) and rate increases supported those reported for most plants, i.e., ranging between two- and fivefold (Jones, 1998). According to Chapin (1980), weak evidence exists that species adapted to infertile conditions are more effective than other species adapted to more fertile environments in increasing nutrient availability at the root surface, as crops and species from both environments have similar rates of root exudation and root phosphatase activity. Lambers and Poorter (2004) shared this view, but emphasized that slow-growing species (as observed for Proteaceae species) may possess mechanisms to release nutrients when these are sparingly soluble.

Oxalate synthesis and exudation might be an adaptive plant strategy to enhance P acquisition as oxalic acid could participate in ligand exchange and dissolution reactions with P bound to Fe and Al oxides (Fox and Comerford, 1992) and is especially suitable for accessing P from Ca-P minerals (Ström et al., 2002). Cannon et al. (1995) suggested that oxalate might be important for regulating P dynamics of long-term plant productivity in tropical ecosystems. Generally, organic acids have low nutrient mobilization efficiency in soils due to rapid binding to the soil surface and microbial breakdown, but oxalate was shown to be more resistant to microbial degradation than citrate and malate (Cannon et al., 1995; Ström et al., 2005). Trolove et al. (2003) reported that oxalate is more efficient than citrate in reducing P sorption from kaolinite, a typical clay mineral of highly weathered acid soils. Carboxylate exudation rates of the two grasses supported literature values of grasses, i.e., 10–50 times lower than for legumes (Gerke et al., 2000). Furthermore, our exudation rates might be underestimated as calculations were made for whole root systems while root exudation might be localized to specific regions of the root system. For example, oxalate exudation by root tips of sugar beet was three times higher than that of the whole root system (Beissner, 1997; Neumann and Römhelt, 2000). Huguenin-Elie et al. (2003) used a modeling approach and reported that low release rates of citrate could account for 80% of total P uptake in rice.

The two grasses differed in the fine-tuning of oxalate secretion as oxalate was the dominant exuded carboxylate in signalgrass and lactate in ruzigrass when plant P concentrations reached critical values of  $0.1 \text{ mg g}^{-1} \text{ DM}$  in low-P grown adult plants. Oxalate was also one of the dominant secreted organic acids in P-deficient elephantgrass, another tropical forage grass that grows well in acid soils (Shen et al., 2001). Oxalate exudation may confer signalgrass with a long-term selective advantage in low-P acid soils as the continuous release of small amounts could solubilize large amounts of P on an annual basis (Fox and Comerford, 1992; Gadd, 1999), while Dong et al. (2004) suggested that oxalate exudation presents a strategy to maximize efficient C utilization.

For ruzigrass, a situation of controlled rhizodeposition as observed for signalgrass may not apply. While lactate is commonly exuded by acid-soil species (Tyler and Ström, 1995), its presence has been linked to detoxification during cytoplasmic acidosis (Neumann and Römhelt, 2000). The total below-ground output of carboxylates and other exudate components such as sugars might be higher for ruzigrass due to high exudation rates when the stimulation of root growth under low P supply is also accounted for; therefore, C costs due to exudation (Dilkes et al., 2004) might be substantial as faster-growing grasses deposit more C than slower-growing ones (Warembourg et al., 2003). Another consideration is that the diversion of C compounds to acquire P is possible when leaf N concentrations are high (Sprent, 1999) and evidently, this is an additional negative aspect for ruzigrass as high RGR species are considered to be more N-demanding (Nagel and Lambers, 2002). In addition, both N and P are limiting nutrients for pasture production in tropical soils (Rao et al., 1998).

Oxalate appears to be a metabolic feature for which the adaptive functionality has wider ecological implications beyond the advantages in terms of P release. A closer consideration of other functions highlights the importance to develop a cost-benefit understanding of key plant traits involved in plant adaptation. Lynch (2007) emphasized for trait-based selection to be effective, we need to understand the biology of these traits, how they contribute to the fitness in the context

of other traits and the potential eco-physiological tradeoffs they may entail. Other features of oxalate include

- Soil additions of oxalate have resulted in positive and negative priming where soil priming refer to the phenomenon that substrate additions to soils may accelerate or retard the mineralization of soil organic matter (Hamer and Marschner, 2005).
- Soil additions of K-oxalate showed potential as nitrification inhibitor as the former did not affect nitrogen fixing capacity of *Azotobacter*, but increased the activity of both oligonitrophilic and cellulose degrading bacteria in Uzbekistanian soils (Mamiew et al., 2002). Interestingly, brachialactone is a diterpene biological nitrification inhibitor exuded by *Brachiaria humidicola* (Subbarao et al., 2009).
- Oxalate exudation might serve as a dual ecological solution for Al toxicity and P deficiency, which are both constraints for *Brachiaria* pasture productivity in tropical soils (Miles et al., 2004). Conyers et al. (2005) reported that  $\text{Al}^{3+}$ -oxalate complexes are the most C-cost-effective method for consuming protons in acidic soils with  $\text{pH} \leq 4.2$ . Wenzl et al. (2001) reported that carboxylate exudation could not be linked to external Al detoxification in both grasses. The mechanisms of Al tolerance have been shown to be affected by P and Al interactions (Gaume et al., 2001b) and responses from the earlier Al studies with the grasses might have been influenced by a low P background as suggested by Liao et al. (2006). Furthermore, hydroponically-grown grasses may be unable to release carboxylates in response to Al (Schöttelndreier et al., 2001), and while equimolar amounts of citrate might be sufficient to reduce Al toxicity in hydroponic systems, two- to threefold excesses of malate and oxalate might be required (Tesfaye et al., 2001).
- Oxalate in root exudates of tomato was inhibitory toward plant growth-promoting *Pseudomonas* strains (Kravchenko et al., 2003).
- Oxalate may participate in facilitative rhizosphere interactions and an antioxidant role in counteracting plant invasiveness was reported by Weir et al. (2006), while Radersma and Grierson (2004) found a role for oxalate for maize grown in agroforestry systems on Ferralsols in the tropics.
- Oxalate is a virulence factor for several phytopathogenic fungi, e.g., oxalate production by *Sclerotinia sclerotiorum* suppressed the oxidative burst of plants (Cessna et al., 2000) and induced foliar wilting as stomata remained open due to  $\text{K}^+$  accumulation in guard cells and through inhibition of abscisic acid-induced stomatal closure (Guimarães and Stotz, 2004).
- Oxalate is an antinutritional factor as it was inhibitory to sucking insects such as *Spodoptera exigua* (Korth et al., 2006) while high plant oxalate content has been associated with the poisoning and death of livestock. Both *B. humidicola* and *B. decumbens* have been reported to contain oxalates that cause big head (parathyroidism) in horses (Thomas, 2004). Low tissue Ca levels might attribute to these observations (Jones and Ford, 1971).

#### 44.4.3 HIGHER RATES OF RELEASE OF CELL-WALL-ASSOCIATED APASES IN P-DEFICIENT RUZIGRASS

Bosse and Kock (1998) reported concomitant increases of phosphatases, phytases, and RNases in P-deficient plants. Low-P grown plants of both species had twofold higher root tissue activities of APases and phytases than high-P grown plants, but absolute values for a specific P treatment did not differ between grasses (Louw-Gaume et al., 2010). Rao et al. (1999b) reported higher root APase activities for *B. dictyoneura* grown in an Oxisol with no added P fertilizer, compared with treatments of Al phosphate, Ca phosphate, phytate and cow manure. Nanamori et al. (2004) reported higher shoot APase activities in the first commercial *Brachiaria* hybrid cv. Mulato (*B. ruziziensis* 44-06  $\times$  *B. brizantha* cv. Marandú) and these enzymes may enhance internal P turnover and P-use efficiency.

Phytase amounts considered relative to the total pool of both internal and external APases represented only minor proportions. Phytase proportions of root tissue varied between 0.6% and 0.7% (Louw-Gaume et al., 2010) and proportions of root exudates were slightly higher, between 2% and 5% (Louw-Gaume, 2009) as reported by Hayes et al. (1999) for other forages. This is not surprising given reports that phytate availability in soils is low and plants possess limited capacity to exude phytases (George et al., 2005). Studies on the effectiveness of phytate, which can be a major component of organic soil P, as P source showed that phytate appears to be poorly utilized by plants (Randall et al., 2001; George et al., 2005).

Temporal patterns of APase release also corresponded closely with the development of P deficiency in both grasses. As observed for oxalate exudation, secreted-APases (one group of APases collected into  $\text{CaCl}_2$  solution) increased in low-P grown ruzigrass 7 days after inducing low P treatment (together with a first decline in plant P concentrations) and both biochemical parameters increased in signalgrass only after 14 days when plant P concentrations declined for the first time. For both grasses, cell-wall-associated APases (another group of APases released into NaCl solution) increased when plant P concentrations declined from 0.2 to 0.1  $\text{mg g}^{-1}$  DM (i.e., after 14 days of low P supply). Secretion rates of cell-wall-associated APases differed significantly between grasses, with threefold higher rates in ruzigrass and this strong induction of cell-wall-associated APases could be associated with effects of P deficiency on leaf and root growth while growth was not affected in signalgrass (Louw-Gaume, 2009), probably due to more optimal photosynthesis at similar plant P concentrations (Fredeen et al., 1989; Rychter and Rao, 2005). Our findings also suggest that molecular events associated with P deficiency get triggered long before the development of visual changes in the phenotype. Thus, these responses are not generalized stress responses, but specific to P stress (Jain et al., 2007). For ruzigrass, cell-wall-associated APases might serve as diagnostic marker of P deficiency (Louw-Gaume, 2009).

However, despite adequate soil amounts of APases, the reaction appears to be substrate-limited (Neumann and Römheld, 2000) and hydrolytic enzymes released by rhizosphere microbes might help to degrade organic matter down into compounds accessible to simple phosphatases, which cleave mainly phosphate mono- and diesters (Troløve et al., 2003). Lefebvre et al. (1990) suggested that external APases may function as part of a salvage system in the hydrolysis of ester P compounds leaked by roots. George et al. (2006) reported that higher phosphatase activities in the rhizosphere were involved in the depletion of organic P from Oxisols containing very low available P.

The release of carboxylates do not only allow for the chelation of  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ , and  $\text{Ca}^{2+}$ , but may cause organic P to become more susceptible to hydrolysis by APases (Bhatti et al., 1998; Vance et al., 2003; Jones et al., 2004; Playsted et al., 2006). Functional synergy between oxalate and APases might be part of a coordinated strategy to access P from unavailable P forms in infertile acid soils. Beissner (1997) reported that oxalate in root exudates can contribute, to some extent, toward phytate mobilization in soils.

#### 44.4.4 PHYSIOLOGY OF OXALATE EXUDATION

##### 44.4.4.1 Leaf and Root Oxalate Concentrations

Preferential root exudation of carboxylates with the highest efficiency in P mobilization under conditions of P limitation appears to be related to increased root biosynthesis of carboxylates and sometimes in shoots. In species with intense exudation of carboxylates during P deficiency, such as oilseed rape, chickpea, and white lupin, organic acids mainly accumulate in root tissue and in root zones where exudation is most intense (Neumann and Römheld, 2007). Vance et al. (2003) reported that carboxylate concentrations of P-deficient roots do not necessarily relate to amounts released as exudates, a finding that suggests selective synthesis of organic acids during P deficiency. Tyler and Ström (1995) confirmed that high foliar oxalate content does not necessarily relate into high amounts of oxalate in root exudates. Carboxylates exist as organic acid anions at the near-neutral

pH typical of the cytosol of plant cells and are released down an electrochemical gradient via anion-permeable channels. Transport across the plasma membrane has been suggested to be the most regulatory step (Ryan et al., 2001; Neumann and Römheld, 2007).

Signalgrass had higher leaf oxalate concentrations (Louw-Gaume, 2009) as reported for slow-growing plants (Libert and Franceschi, 1987). Oxalate concentrations of leaves were higher than that of roots in both grasses; and between grasses, signalgrass had higher leaf-to-root oxalate ratios due to higher leaf and lower root concentrations. When P stress became more severe, only shoot oxalate concentrations declined in signalgrass while for ruzigrass, shoot and root concentrations decreased (Louw-Gaume, 2009). Thus, leaves might be the sites of oxalate biosynthesis as found by Ji and Peng (2005), followed by shoot-to-root transfer. Jeschke et al. (1997) suggested that leaf vacuoles might have greater storage capacity for carboxylates in P-deficient *Ricinus communis* L. Oxalate (and fumarate) was also detected in leaves of both P-sufficient and severely P-deficient plants of *Brachiaria* hybrid cv. Mulato (Nanamori et al., 2004), highlighting the importance to understand the biosynthesis and regulation of oxalate as a central carboxylate associated with P-efficiency mechanisms of *Brachiaria* grasses.

#### 44.4.4.2 Oxalate Biosynthesis

Glycolate appears to be an important oxalate precursor as plant glycolate concentrations were significantly higher, up to 30-fold, than for oxalate in low-P grown plants of both grasses (Louw-Gaume, 2009). Oxalate can be formed through the oxidation of photorespiratory glyoxylate via glycolate catalyzed by glycolate oxidase (Franceschi and Nakata, 2005). The possibility of glycolate as oxalate precursor in *Brachiaria* grasses is further supported by Ueno et al. (2005) showing higher glycolate oxidase activities in *B. decumbens* (and *B. brizantha*) among a total of 28 C<sub>4</sub> grasses tested. Furthermore, temporal patterns of oxalate and glycolate revealed an inverse relationship as decreasing leaf oxalate concentrations during the development of P deficiency could be associated with increasing leaf glycolate concentrations (Louw-Gaume, 2009). However, various oxalate biosynthetic pathways might be operating at the same time in a single plant (Raven et al., 1982), e.g., in rice, oxalate accumulation was shown to be independent of glycolate oxidase (Xu et al., 2006). In addition, ascorbic acid is another biosynthetic precursor of oxalate, especially in species with high Ca oxalate contents (Franceschi and Nakata, 2005).

#### 44.4.4.3 Mechanisms of P Deficiency–Induced Oxalate Exudation: The Role of Counter-Ions

Carboxylate anion release necessitates the counter release or uptake of a cation or an anion, respectively, to maintain electroneutrality. In the case of P deficiency, the counter-ion of organic acid anion release is unknown, but in some plants, the rhizosphere pH declined concurrently with carboxylate release, suggesting a balancing role for proton efflux via H<sup>+</sup>-ATPases (Hinsinger et al., 2003).

Shane and Lambers (2005) commented that it remains to be established whether H<sup>+</sup> exudation can really be regarded as an adaptation for improved P acquisition or whether it is simply a component of the mechanisms for carboxylate export, which may partly be substituted by other cations such as K<sup>+</sup>. Our studies confirmed that the interpretation of pH changes in the rhizosphere should be done with caution (Louw-Gaume, 2009). While the pH of nutrient solutions with plants decreased over time, with a stronger response for ruzigrass, pH measurements of root exudate-containing CaCl<sub>2</sub> solutions increased, and this response might be attributed to reduced Ca<sup>2+</sup> uptake as suggested by Hinsinger et al. (2003). Low-P grown plants of both species did not differ remarkably and the capacity to increase the pH of CaCl<sub>2</sub> solutions diminished over time as plants grew bigger. Proton fluxes may also be related to differential uptake of cations/anions and this might explain the increase in the rhizosphere pH by *B. dictyoneura* (Hylander and Ae, 1999) and the increase in acidity by *B. humidicola* and *B. brizantha* (Logan et al., 2000).

Coupling of malate and citrate release to K<sup>+</sup> efflux was reported in plants (Ryan et al., 2001; Zhu et al., 2005; Lambers et al., 2006) while Palomo et al. (2006) showed that alkalinization of

the rhizosphere by K-citrate results in enhanced P mobilization in an acid soil with high P sorption capacity. Potassium efflux increased strongly over time in low-P grown plants of both grasses, but an association with the oxalate response was not evident for signalgrass. An increase in K<sup>+</sup> efflux was accompanied by declining root K<sup>+</sup> concentrations in P deficient plants (Louw-Gaume, 2009). Lower root K levels under low P conditions were reported for *Lupinus* (Sas et al., 2002) and the *Brachiaria* hybrid cv. “Mulato” (Watanabe et al., 2006). Interest in K relations is important for several reasons:

- K or Na salt of oxalate is predominantly found in grasses (Jones and Ford, 1971).
- K functions in charge balance, especially in nitrate-fed plants (as in our studies).
- K participates in phloem transport of organic acids and soluble sugars (Marschner et al., 1997).

For both grasses, Mg<sup>2+</sup> could be a counter-ion involved in charge balance during oxalate exudation (Louw-Gaume, 2009) as reported by Zhu et al. (2005) in P-deficient white lupin. Other rhizosphere functions for Mg<sup>2+</sup> may be to increase Ca-oxalate solubility in soils with pH values below 5 (Gadd, 1999) and to aid in organic acid uptake by microorganisms (Jones et al., 1986). Efflux patterns of Mg<sup>2+</sup> corresponded in a timely manner with decreases in root Mg concentrations in both grasses (Louw-Gaume, 2009).

Under P deficiency, the lipid composition of thylakoid membranes may also display decreases in phospholipid and increases in sulfolipid amounts (Vance et al., 2003) and thus, membrane leakiness might contribute to nonspecific efflux of organic acids and counter ions.

44.4.5 NITRATE EFFLUX, MALATE TISSUE CONCENTRATIONS, AND Pi HOMEOSTASIS

Temporal patterns of nitrate (NO<sub>3</sub><sup>-</sup>) concentrations in root exudate solutions of low-P grown plants showed that NO<sub>3</sub><sup>-</sup> was released by both grasses. In ruzigrass, NO<sub>3</sub><sup>-</sup> efflux leveled off when plant P concentrations decreased from 0.2 to 0.1 mg P g<sup>-1</sup> DM (Louw-Gaume, 2009), but increased in signalgrass. Nagel and Lambers (2002) reported higher NO<sub>3</sub><sup>-</sup> efflux rates in slow-growing species. Inadequate P can reduce NO<sub>3</sub><sup>-</sup> uptake through feedback inhibition and alterations to energy metabolism (Rufty et al., 1993; Jeschke et al., 1997; Gniazdowska and Rychter, 2000); e.g., tissue malate levels have been shown to control nitrate uptake. High NO<sub>3</sub><sup>-</sup> uptake rates were associated with high leaf biosynthesis and fast shoot-to-root translocation of malate (Martinoia and Rentsch, 1994). Table 44.3 shows that the regulation of NO<sub>3</sub><sup>-</sup> uptake by malate concentrations and phloem translocation rates might be more critical for ruzigrass than for signalgrass as leaf concentrations

**TABLE 44.3**  
**Tissue Concentrations of Malate in Hydroponically-Grown Signalgrass and Ruzigrass Determined at Day 7 (D7), Day 14 (D14), and Day 21 (D21) after Inducing Low P Treatment**

| Malate (nmol g <sup>-1</sup> FM) | Harvest | Signalgrass     |                 | Ruzigrass       |                 |
|----------------------------------|---------|-----------------|-----------------|-----------------|-----------------|
|                                  |         | Leaf            | Root            | Leaf            | Root            |
|                                  | D7      | 36 <sup>a</sup> | 23 <sup>a</sup> | 48 <sup>b</sup> | 46 <sup>b</sup> |
|                                  | D14     | 24 <sup>a</sup> | 23 <sup>a</sup> | 14 <sup>b</sup> | 78 <sup>b</sup> |
|                                  | D21     | 32 <sup>a</sup> | 20 <sup>a</sup> | 12 <sup>b</sup> | 31 <sup>b</sup> |

*Note:* Small letters indicate significant differences between grasses at a specific harvest. Experimental conditions are described by Louw-Gaume (2009).

declined together with the first decrease in plant P concentrations, i.e., after day 7 (Louw-Gaume, 2009), while root concentrations (as found for oxalate and glycolate) fluctuated over time. These findings also provide insight into the reduction in leaf growth after day 14 in low-P grown ruzigrass. Species with a high RGR generally allocate a large fraction of N to their leaves, and have higher rates of photosynthesis (Nagel and Lambers, 2002) and thus, P deficiency could have affected photosynthetic rates in ruzigrass by down-regulation of  $\text{NO}_3^-$  uptake.

Malate functions as a hydrogen carrier in balancing cellular energy supplies (Scheibe, 2004), suggesting that limiting P availability for growth affected energy metabolism in ruzigrass as well as activities of enzymes, such as phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase, that are associated with the biosynthesis and maintenance of root malate concentrations (Tesfaye et al., 2001; Vance et al., 2003). Interestingly, leaf and root PEPC activities increased in the *Brachiaria* hybrid cv. Mulato in response to P deficiency (Begum et al., 2006).

Root malate concentrations (as found for oxalate and glycolate) did not show strong temporal variation despite decreasing plant P concentrations in low-P grown signalgrass (Table 44.2), illustrating that physiological mechanisms responsible for  $\text{P}_i$  homeostasis and energy provision might be different between grasses. Phosphate homeostasis is achieved by a combination of membrane transport and exchange between various intracellular pools of P. Plaxton and Carswell (1999) reported that during early plant-P starvation responses, cytoplasmic  $\text{P}_i$  is maintained at the expense of non-metabolic vacuolar  $\text{P}_i$  reserve, but that prolonged starvation eventually depletes  $\text{P}_i$  stores and cytoplasmic free  $\text{P}_i$ , nucleotide-P and other P-metabolite pools decrease. Vacuolar  $\text{P}_i$  stores can fluctuate dramatically and may serve an important sensor for P deficiency-induced responses. Labeling studies with P isotopes could elucidate the nature and distribution of P compounds while  $^{31}\text{P}$ -NMR studies could contribute to map intracellular P pools (Schachtman et al., 1998). The P pools may vary according to

- Location in physical compartments such as the cytoplasm, vacuole, apoplast, and nucleus while the pH of these compartments.
- Chemical form of P, e.g., as  $\text{P}_i$ , P-esters, P-lipids, and nucleic acids.
- Physiological function, e.g., as metabolic, stored, and cycling forms.

Elucidation of the contribution of the citric acid cycle, its intermediates and enzymes may be important to dissect the plethora of responses induced by P limitation in *Brachiaria* grasses.

#### 44.5 MORPHOLOGICAL PLASTICITY HELPS RUZIGRASS TO COPE WITH LOW P SUPPLY

It is evident that although certain traits are conserved in a wide variety of species from different environments, they are by no means identical in all plants. The morphological and physiological trait profile of signalgrass suggests that traits associated with environmental fitness might be of greater significance for persistence on low fertility acid soils. The embedded overall advantage is that these traits might contribute to high field root densities to prolong seasonal growth and to ensure that a strong feedback loop on shoot growth is formed as suggested by Chapin (1980). Specific attributes of signalgrass are as follows:

- Signalgrass is a slower-growing grass, and species with slow growth rates are more tolerant to low nutrient supply and less responsive to increased nutrient variability.
- Higher shoot mass densities might reduce nutrient losses, increase resistance to a range of environmental hazards and increase the yield of photosynthate per unit of leaf P.
- Signalgrass expressed developmental homeostasis at root morphological and root physiological level as lateral root growth was maintained in response to variation in P supply and root tissue levels of carboxylates were maintained despite decreasing plant P concentrations.

- Physiological mechanisms such as plasticity in nutrient partitioning between main and lateral roots and the dominance of oxalate in root exudates might present an ecological strategy that contributes to regulate not only P uptake (Lambers and Poorter, 2004), but also N uptake (in case it functions as nitrification inhibitor), to optimize internal P recycling (Shane and Lambers, 2005) and to ensure plant re-growth upon defoliation (Boot and Mensink, 1990).

It is important to consider the disadvantages associated with a slower growth rate for *Brachiaria* grasses as the most planted tropical forage grasses in the world. Adaptation to nutrient-poor environments by minimizing nutrient losses also have negative side effects as reduced nutrient productivity, higher biosynthesis costs of tissues, and reduced potential growth are disadvantages when soil fertility increases.

Our comparative analyses between signalgrass and ruzigrass suggest that selection for ruzigrass might have been in soils of higher soil fertility, as suggested by Miles et al. (2004). Chapin and Bielecki (1982) also proposed stronger selection for high absorption capacity in moderately fertile soils where P diffusion is less limiting. For ruzigrass, the long-term disadvantage of a faster growth rate in tropical acid soils can be due to the short lifespan of low density tissue that is required for rapid tissue expansion. Apparently, evolution has not allowed for maximum RGR and low nutrient-loss rates to be combined and hence, plant properties that determine nutrient losses and potential growth rates are strongly interconnected and the low maximum RGR-low nutrient loss rate and high maximum RGR-high nutrient loss rate strongly correspond, respectively, with the stress-tolerant and competitive strategies of plants (Grime, 1977; Chapin et al., 1993; Berendse et al., 1999).

To cope with P limitation, ruzigrass relied on similar phenotypic traits and biochemical mechanisms as described for signalgrass, but also on a higher degree of morphological plasticity as evident from the increases in lateral root growth and leaf mass density when P availability for growth was limiting (Louw-Gaume, 2009; Louw-Gaume et al., 2010). Robinson and Rorison (1988) highlighted that plasticity compensates for the constraints imposed by an environment on plant fitness, but morphological plasticity is also considered to be a high-cost ecological solution and not sustainable for slow-growers that are adapted to less productive natural environments (Hutchings and De Kroon, 1994). Phenotypic plasticity is expressed continuously and forms an integral part of all plants (Grime and Mackey, 2002) and plant traits such as biomass production, biomass allocation, anatomy, and chemical composition are a result of genetic as well as environmental influences (Wahl, 2000). Howe and Brunner (2005) summarized the viewpoint of evolutionary ecologists who agree that plant adaptations are phenotypic traits favored by natural selection while phenotypic plasticity is as important as real genetic adaptation.

Wissuwa (2003), using a modeling approach, concluded that improving root growth is the most efficient way to enhance tolerance to P deficiency in rice. Plasticity to increase lateral root growth when P availability decreases might allow ruzigrass to cross geographic distribution areas as suggested by Valladares et al. (2007) and to grow in less fertile soils. However, this adaptive response in root growth was associated with a strong reduction in stem growth (Louw-Gaume et al., 2010) while both Luquet et al. (2002) and Wissuwa et al. (2005) argued that pronounced increases in R:S ratios where high root growth rates could not provide positive feedback on shoot growth are more likely a sign of low-P intolerance. It is difficult to envisage that the same long-lasting benefits of high field root densities and higher C-use efficiency as suggested for signalgrass could help ruzigrass to survive in low-P acid soils.

Craine (2006) stated that the allocation of resources between roots and shoots should be subject to strong natural selection in order to optimize growth and fitness, but whether plants allocate optimally is questionable. In this regard, cost-benefit approaches are advocated as a means to assess optimal allocation rates for plants that account for both the nutrient under investigation as well as C (Lynch and Ho, 2005). The processes linking root P starvation responses to those involving C was reviewed by Hammond and White (2008) and include differences in sugar and starch accumulation

in leaves, carbohydrate transport to roots and associated plant-derived signals. Liu et al. (2005) suggested that the systemic signal for the shoot P status might be transduced via sugars and sugar phosphates.

## 44.6 FUTURE PERSPECTIVES

The adoption of agro-ecophysiological strategies to elucidate the physiological basis of low-P adaptation in signalgrass and ruzigrass permitted the development of a whole plant perspective and to link results obtained in the field with those from controlled growth chambers.

Gutschick and Pushnik (2005) suggested that plants selected for agricultural purposes yet capable of growing in nutrient-poor soils may have diverged from the fitness functions of wild plants and have a somewhat different set of nutritional properties. Our results suggest that the selection of *Brachiaria* grasses for extensive agricultural systems should be based on plant traits associated with plant survival and persistence that are observed with signalgrass while nutrient-responsive *Brachiaria* hybrids with traits that are found in ruzigrass might be suitable for smallholder systems where cut-and-carry systems in which productive potential for higher forage yield is desired for livestock production.

### 44.6.1 RECOMMENDATIONS FOR BREEDING AND SELECTION OF *BRACHIARIA* GENOTYPES

A combination of morphological, physiological, and biochemical traits may be promising as selection criteria for the ongoing *Brachiaria* breeding program:

- First, biomass production in combination with shoot tissue mass density
- Second, lateral-to-main root proportions
- Third, root carboxylate concentrations
- Fourth, the combination of oxalate-to-lactate ratios and cell-wall-associated APase activities in root exudates

The power of the first two suggestions stems from observations that grasses differed with regard to these traits irrespective of plant-P status. Potential advantages are that the collection of shoot and root material is not difficult and the methodology does not require too much resources. A recommendation is to include screening under rather high nutrient supply conditions as faster growing genotypes will be more nutrient responsive, show a strong response in biomass production, have lower shoot mass densities, and show reduced lateral root growth. It is anticipated that these screening criteria could deliver successful outputs for studies in nutrient solution as well as in sand/soil systems. For root studies in sand/soil systems, it will be important to ensure that root growth is not limited by pot size.

In fact, biomass production has always been a key criterion for the selection of *Brachiaria* genotypes (John Miles CIAT, personal communication) and thus, the proposed strategy fits into the current methodologies used by CIAT. The investigation of biomass accumulation in combination with tissue mass density, a parameter considered to explain variation in ecosystem nutrient-use efficiency (Lambers et al., 1998), might add value to the current paradigm of *Brachiaria* breeding and potentially that of many other forage-breeding programs. Lynch (2007) suggested that the second Green Revolution will be based on crops tolerant to low soil fertility and thus, a consideration for breeding programs could be to incorporate the tradeoff between traits linked to strategies as described for wild plants (e.g., survival/persistence and signalgrass is an example among *Brachiaria* cultivars) and those more similar for crop plants (e.g., high nutrient responsiveness and ruzigrass provides a suitable model). Differences in tissue mass density and therefore, differences in secondary metabolites such as lignins and tannins are also important for studies concerning the feeding value of *Brachiaria* genotypes (Lascano and Euclides, 1996).



A kinetic approach in combination with the monitoring of plant P concentrations will be required if the last two proposals are implemented for plant selection. Our studies demonstrated the value of temporal approaches and thus, the inclusion of multiple harvests, to follow the sequence of morphological and biochemical events that unfold during the development of plant-P deficiency (Louw-Gaume, 2009). This strategy also contributed to more precise phenotypic characterization of *Brachiaria* grasses in the studies of Kerguelén et al. (2009). However, methodologies targeted at differentiating between root exudation strategies in association with root tissue concentrations of organic acids will be more time consuming and require sophisticated instrumentation and skilled technicians.

Hydroponic experiments provide only indirect evidence and the functional significance of root exudation components in a real soil environment remains unknown and needs to be confirmed (Jones et al., 2004). Dessureault-Rompré et al. (2006) showed the value of micro suction cups in a rhizobox system to follow in situ exudation and found higher concentrations of organic acids in the rhizosphere soil solution of active cluster roots of white lupin than in the bulk soil solution.

Plant material used in the present study resulted from seeds. As the seed production of a large number of new *Brachiaria* hybrids has been shown to be problematic (Miles et al., 2004), the practice of stem cuttings (vegetative propagules) was adopted by CIAT for screening experiments (Ishitani et al., 2004; Wenzl et al., 2006). A note of caution for extrapolating our findings to their use as future selection criteria is to standardize these stem cuttings in terms of size, rooting potential, tissue mass density, and nutrient concentrations.

Randall et al. (2001) noted that progress toward improving P acquisition may be slow using selection criteria based on plant performance, because many factors are involved, they interact and their relative importance changes with P supply. Root morphological characters, although difficult, has given promising results, but there has been less interest in selection on the basis of root function, largely due to a lack of information that can be used to identify suitable selection criteria. Helyar (1998) also reported that the ability of plants to use sparingly soluble forms of P may be so universal and this trend toward convergent evolution may not be surprising given the widespread existence of P-deficient soils in nature. Furthermore, Marschner (1998) warned that plant selection based on a few components is unlikely to be successful and a study of nutrient efficiency as a dynamic process at different sites, measuring both nutrient uptake and yield is more appropriate. Despite these notes of caution, the implementation of the complete package of suggestions should be the ultimate goal of efforts directed toward the improvement of criteria suitable for the breeding of P-efficient *Brachiaria* genotypes. The examination of multiple parameters and the adoption of trade-off approaches to pinpoint the real significance of physiological responses and interactions of P with other nutrients such as C could enhance the chance to detect the “true” adapted genotypes.

#### **44.6.2 NEED FOR MULTIDISCIPLINARY APPROACHES TO UNDERSTAND PLANT GROWTH RESPONSES TO BIOTIC AND ABIOTIC STRESS FACTORS**

Investigations into morpho-physiological and biochemical mechanisms of P efficiency that could explain differences in field persistence between signalgrass and ruzigrass suggest that the key to the detection of subtle changes in growth is to be familiar with whole plant development and plasticity responses to P stress. In addition, plant adaptation to a specific soil constraint should not only be considered in terms of specific mechanisms strongly supported by literature, but should include reference to resource-use strategies and thus, ecological strategies including evolutionary history of the genotypes under investigation. A broader view provided by the implementation of multidisciplinary approaches based on the crossing of the disciplines of crop science, plant physiology, and ecology might be important if future attempts to breed for nutrient efficient plants are expected to succeed. Tilman (1998) highlighted this aspect stating that “the principles of ecology, epidemiology, evolution, microbiology, and soil science operate in agroecosystems as well as in natural systems.”

Brewster et al. (1975) suggested that the connection between uptake rates, growth rates, and internal nutrient concentrations and the way these processes are internally controlled by plants requires more investigation if a sounder basis for growth models is to be found. This proposal still appears to be valid. BassiriRad (2005) highlighted that progress was made during the past 30 years and the mechanisms that control plant-nutrient uptake and how plants respond to changes in the environment contributed to our understanding of the forces that govern today's plant communities, and are indispensable to models designed to predict how plant communities will respond to projected changes in the global climate.

Rao et al. (1999c) indicated that it is possible to envisage situations in which the improved adaptation of forage grasses to major abiotic constraints combined with improved adaptation to major biotic constraints and improved nutritional quality could have a tremendous impact on food security and human nutrition in the tropics. In this regard, Cakmak (2002) emphasized that plant nutrition research is a major promising area in meeting the global demand for sufficient food production with enhanced nutritional value in this millennium.

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# 45 Forgotten Link in Improving Crop Salt Tolerance under Brackish Irrigation: Lateral Soil Salinity Gradients around Roots

*Uwe Schleiff*

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## 45.1 INTRODUCTION

Most crops cultivated for human or animal consumption suffer severely under saline soil conditions. Leaves may change their color. The youngest one may look normal green, older ones darker green, but the oldest look silvery and edges become progressively necrotic. However, it is not only the shoot that reflects saline growth conditions, it is also the root. From field observations it is well known that, for example, roots of sweet potatoes growing under saline soils conditions develop a much finer root system than under nonsaline conditions. So, we conclude that modifications at root level are also a strategy of plants to adapt to saline soil conditions and to improve their salt tolerance (Schleiff, 2005).

## 45.2 ANALYSIS OF PRESENT CONCEPT FOR CROP SALT TOLERANCE RATING

The present concept internationally applied for crop salt tolerance rating is soil based and considers the vertical salt distribution under controlled irrigation in a soil profile (Maas and Hoffmann, 1977; Ayers and Westcot, 1985). As shown in Figure 45.1, the total rooted soil layer is divided into four layers (25% each) of differing rooting densities, which contribute different parts to the total water supply of the plant. It is generally assumed that the upper quarter of the rooted soil covers 40%

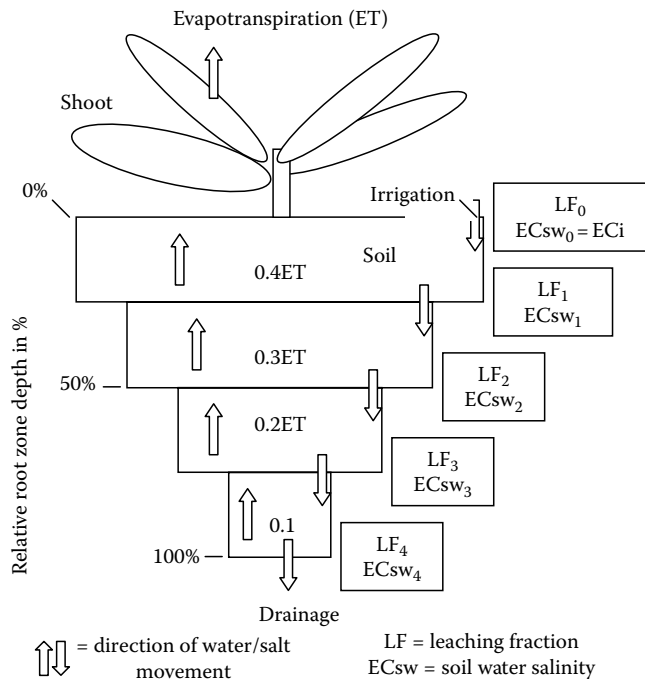


FIGURE 45.1 Basics of present concept for crop salt tolerance rating.

(0.4ET) of the evapotranspiration (ET) demand, but decreases continuously to 10% (0.1ET) toward the bottom layer. Consequently, the amount of water available for salt leaching (LF=leaching fraction) downwards the soil profile decreases with profile depth.

As demonstrated in Figure 45.2, soil salinity profiles are affected by different leaching LFs. Under irrigation with a brackish water containing  $EC_i$  of 5 dS/m, the soil water salinity ( $EC_{sw}$ ) increases from 5 dS/m at the surface to about 12 dS/m at the bottom at a LF of 0.4, but to 20 dS/m at a lower LF of 0.25. Additionally, the average EC of soil saturation extract (ECe) of the root-zone can be calculated, which serves to estimate yield losses for a specific crop. Modifications of this

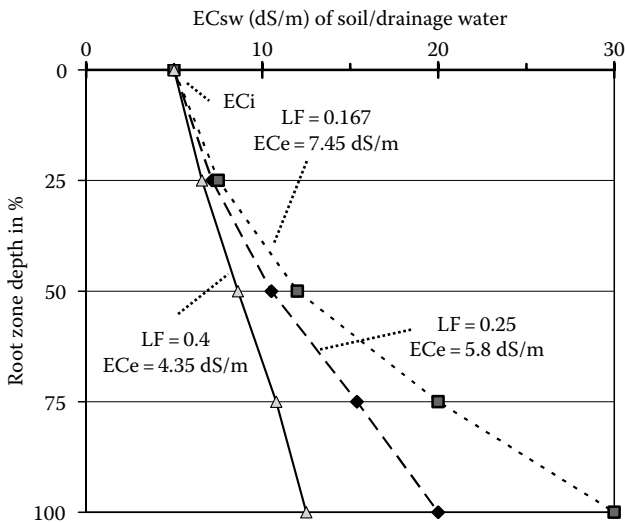
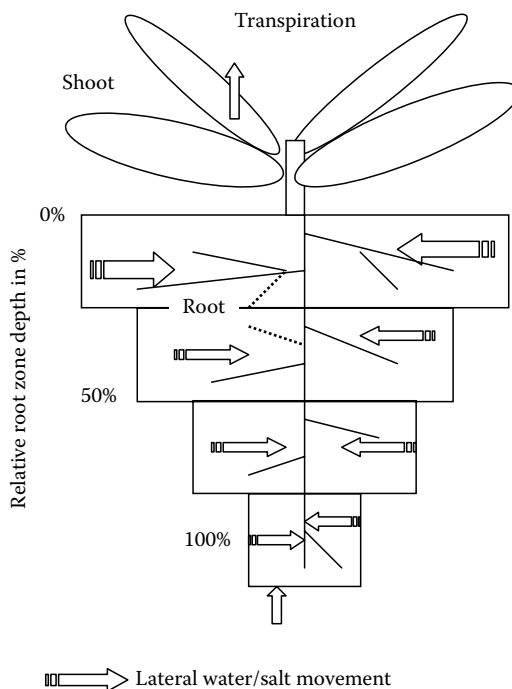


FIGURE 45.2 Outcome from applications of the salt tolerance rating concept.



**FIGURE 45.3** The “forgotten” link: impact of lateral water and salt movement.

concept are principally possible for specific applications. However, in this context there is no need to go deeper into details as this concept is well known for decades (Rhoades et al., 1992 and Pasternak and de Malach, 1995).

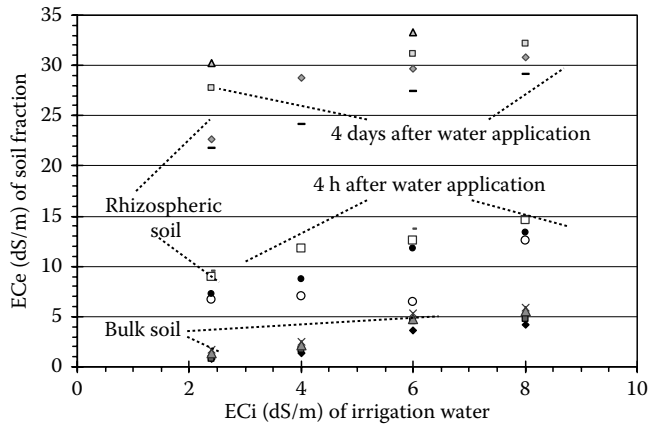
The focus of the presented concept is the vertical salt and water movement, which predominates at soil water contents exceeding field capacity and results in typical soil salinity profiles. It does not take into account the lateral movement of soil water and salts, which predominates during periods of soil water uptake by plant roots following a water application (Figure 45.3). Leaf transpiration is the driving force that initiates the lateral flow of water from the bulk soil into the rhizospheric soil surrounding the root surface (rhizospheric soil: part of rooted soil volume directly altered by roots as compared to the bulk soil). Water and most nutrients are taken up by roots, whereas most salts are excluded from uptake. Consequently, during periods of water depletion, salts accumulate in the rhizospheric soil solution contacting the roots at simultaneously decreasing soil water contents (Schleiff, 1982).

### 45.3 RECENT FINDINGS ON LATERAL SOIL SALINITY GRADIENTS

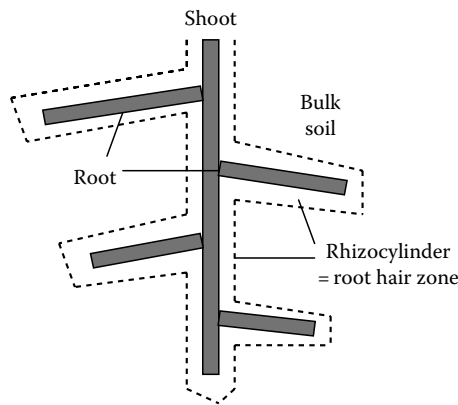
Riley and Barber (1970) and Sinha and Singh (1976) were the first who described a transpiration driven accumulation of easily soluble salts around roots of soybeans and maize. Jungk (2002) visualized this process by irrigating plants with water containing calcium ( $\text{Ca}^{++}$ ) and sulfate ( $\text{SO}_4^{--}$ ) ions. In the soil fraction surrounding the root, he watched the formation of gypsum crystals that develop in water solutions exceeding concentrations of 2 g/L. Illustration of this process with chloride ( $\text{Cl}^-$ ) salts is difficult due to their high water solubility.

In field experiments with onions in Saudi Arabia using brackish waters of different salinities (2.4–8.0 dS/m), we demonstrated that bulk soil and rhizospheric soil salinity differed significantly (Figure 45.4).

It is shown that lowest soil salinity was found in the bulk soil ranging from about  $\text{ECe}$  1.5 dS/m under fresh water ( $\text{ECi}$  2.3 dS/m) up to an  $\text{ECe}$  around 5 dS/m under the highest water salinity ( $\text{ECi}$  8 dS/m). Rhizospheric soil salinity always was significantly higher. Immediately after a water



**FIGURE 45.4** Salinity (ECe) of bulk and rhizospheric soil under brackish irrigation.



**FIGURE 45.5** Schematic illustration of root hair zone respectively volume of rhizocylinder.

application of 50 mm (4 h later), it was still 3–4-fold higher as compared to the bulk soil ranging between ECe of 7–13 dS/m. However, 4 days later, toward the end of the water depletion period, a further increase of the rhizospheric soil salinity to average ECe values in the range of 25–30 dS/m occurred (Schleiff, 1981).

Based on these findings, we concluded that there is a principal need to divide the rooted soil into two soil fractions (Figure 45.5): the bulk soil far from roots that is not directly root affected, and the rhizospheric soil, which is the rhizocylinder volume including the root hair zone and directly root affected, where salts excluded from root uptake accumulate (Schleiff, 2008).

## 45.4 ROOT MORPHOLOGY AS RELATED TO EFFICIENCY OF ROOT WATER UPTAKE FROM SALINE SOILS

### 45.4.1 EFFICIENCY OF NUTRIENT UPTAKE AS AFFECTED BY ROOT MORPHOLOGY

From several experiments in the field of plant nutrition, it is well known that plants differ significantly in their ability to form rhizocylinder volumes. This feature is important as it is often closely related to the efficiency in nutrient uptake, for example, phosphate, potassium, and nitrate uptake (Barber, 1979; Jungk et al. 1982; Kovar and Claassen, 2005). As shown in Table 45.1, roots of leek and rape are known to form rhizocylinder volumes that differ extremely. Therefore, they are well suitable as model plants to analyze effects related to root morphological aspects.

**TABLE 45.1**  
**Morphologic Features of Leek/Onion**  
**and Rape Roots**

| Feature                                           | Onion/Leek | Rape        |
|---------------------------------------------------|------------|-------------|
| Root length, m/g DM                               | 30         | 200         |
| Root hair cylinder, mm <sup>3</sup> /cm           | 4          | 62          |
| <b>Root hair volume, cm<sup>3</sup>/g DM root</b> | <b>12</b>  | <b>1240</b> |
| Root radius, mm                                   | 0.225      | —           |
| Root hair length, mm                              | 0.04       | —           |
| Root hair density, number/cm                      | 1180       | —           |
| K-uptake rate, $\mu\text{mol}/\text{cm}/\text{s}$ | 0.01       | 0.52        |
| K-uptake rate, mmol/g (root-DM)/s                 | 0.03       | 10.4        |

Based on differences in root length and root hair length, leek roots are known to form a small rhizocylinder volume of only 12 cm<sup>3</sup>/g root dry matter (DM). Rape is equipped with roots that may form a 100-fold larger rhizocylinder volume achieving about 1200 cm<sup>3</sup>/g root DM due to their finer roots and longer root hairs. Simultaneously, potassium (K) uptake rates by rape roots were in the order of magnitude 50-fold higher as compared to onion/leek roots (Jungk et al. 1982). Taking these results on efficiency of nutrient uptake by roots differing in morphological properties as a basis, we should ask if they are also relevant for efficiency of water uptake by roots under saline soils conditions. To answer this question, a pot experiment was carried out in order to compare water uptake rates of leek and rape roots under saline soil conditions.

#### 45.4.2 WATER UPTAKE BY LEEK AND RAPE ROOTS FROM SALINE SOILS

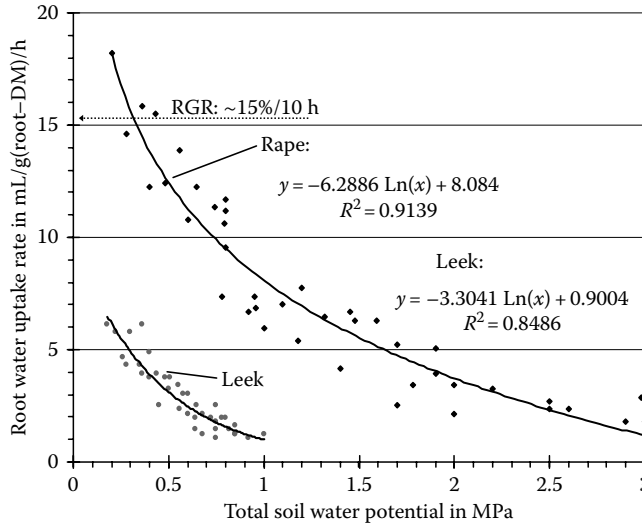
##### 45.4.2.1 Experimental Setup

The experiment was carried out in 300 mL pots with rape (*Brassica napus*) and leek (*Allium porrum*). Pots were filled with 360 g of a dried, silty soil (31% sand, 60% silt, and 9% clay). The moisture profile was held at soil water potentials of -0.1, -10, -35, -70, -200, -500, -1000, and -1500 kPa were 39, 34, 31, 29, 15, 10, 8, and 6 w/w%, respectively, at 47, 41, 37, 35, 18, 12, 10 and 7 vol.%. Soil salinity was <0.5 dS/m in the E<sub>ce</sub>, cation exchange capacity (CEC) 6 meq/100 g soil, with initial soil-pH 7.2. Before sowing, the soil was wetted with 110 mL nutrient solution (Hoagland and Arnon, 1950) to achieve a soil water content of 30.5 w/w% (36.6 vol.%). Evaporation losses from the pot surface were reduced to less than 1 mL/d per pot by covering all pots with a lid, which was pierced with a small opening for the shoot stem only.

The experiment was divided into two phases, a precultivation period of 5 weeks for rape and 10 weeks for leek. The objective of this phase was to achieve a high rooting density of the soil. During precultivation period, rape plants were supplied with 380 mL/pot water in total, leek with 530 mL. The well-controlled water supply contributed to achieve plants that appeared very homogeneous at the end of the precultivation period. In order to adapt plants to slight soil salinity and to avoid sodium (Na) induced calcium (Ca) problems, the water of the last 100 mL-application was salinized with 20 mmol/L NaCl and 20 mmol/L CaSO<sub>4</sub>.

At the end of the precultivation phase, 2 representative rape and leek plants were harvested in order to obtain some plant parameter at the start of the experimental phase. Rape plants had a shoot DM weight of 0.83 g/plant and root DM weight of 0.49 g/plant, which results in a shoot/root ratio of 1.7. The transpiration coefficient (380 mL/0.83 g shoot-DM) was 460 mL/g DM. DM based root density was 1.63 mg/cm soil in average.

Leek plants developed somewhat less homogenous than rape plants. Shoot weight of single plants ranged from 1.1 to 1.4 g/plant and root weight from 0.6 to 0.8 g/plant resulting in a shoot/root ratio



**FIGURE 45.6** Efficiency of water uptake by roots of leek and rape from saline soils.

of 1.8. The average transpiration coefficient (530 mL/1.25 shoot DM) was about 430 mL/g DM, and DM-based root density was 2.3 mg/cm<sup>3</sup> soil in average. Precultivation phase ceased when soil water content has dropped to about 10 vol.%, which corresponded to a soil matric potential ( $\Psi_m$ ) of  $-1.0$  MPa. The experimental phase began with the irrigation of all pots with 90 mL water up to a soil water content of 40 vol.% ( $\Psi_m = -0.01$  MPa), close to field capacity. In order to expose roots to varying salinity stress, water was salinized with NaCl to achieve concentrations of 25 ( $\Psi_o \sim -0.1$  MPa), 50, 100, 150, 200, and 250 mmol/L (2 pots/treatment). Plants were left overnight with no light at 20°C air temperature in a calm room in order to attain a homogeneous water and salt distribution in the soil. During the following days, plants were exposed for 14 h/d to optimal growth conditions in a growth chamber, and pot water losses were measured bi-hourly until severe plant wilting. Soil water potentials were determined from the measured water losses and related to the root masses. For further details, the readers are referred to Schleiff (1987).

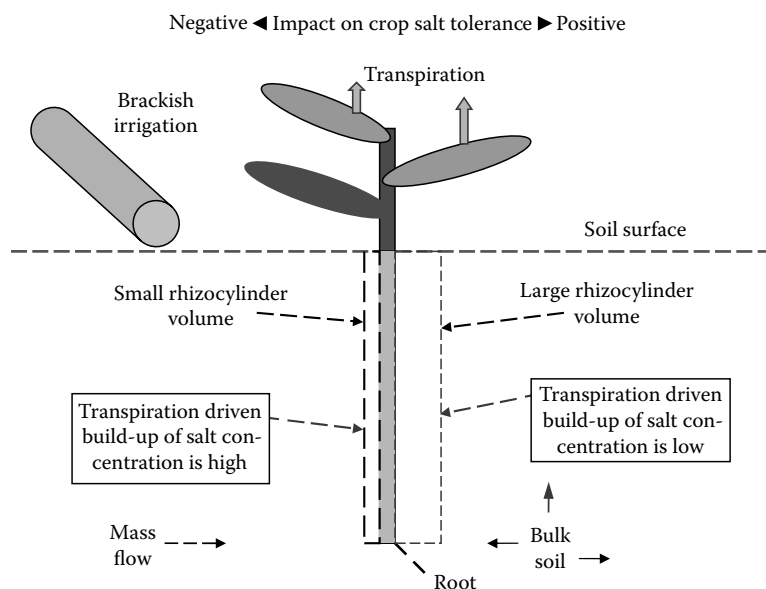
#### 45.4.2.2 Results

Experimental results are presented in Figure 45.6, where water uptake rates by roots of leek and rape are related to the soil salinity expressed as soil water potential ( $\Psi_T$ ). It is clearly shown that at the beginning of the experimental phase, when  $\Psi_T$ -values were highest ( $-0.1$  to  $-0.2$  MPa) and soil water contents near field capacity, water uptake rates by rape roots were nearly 300% higher as compared to leek roots. At higher salinity stress around a  $\Psi_T$ -value of  $-0.7$  MPa, water uptake rate by rape was even 400%–500% higher. Leek roots nearly ceased water uptake from a soil of around  $-1.0$  MPa  $\Psi_T$ , but the water uptake by rape roots was still significant and ceased only at  $< -2.5$  MPa.

### 45.5 DISCUSSION AND RECOMMENDATIONS

In the past decades, much research has been undertaken to extend our understanding on physiological and biochemical processes at cell, organ, and whole plant level associated with salinity stress, always hoping to find a key factor that alleviates salt stress. Flowers (2004), Yamaguchi and Blumwald (2005), Munns (2007), and Munns and Tester (2008) have recently reviewed important findings and trends, which will not be repeated here. It is the objective of this chapter to add an aspect that has been neglected in research for crop salt tolerance enhancement of the last decades, the processes happening between soils and roots at the soil/root interface and their relevance for crop growth in saline soils. The presented results are only a first step indicating that an improved





**FIGURE 45.7** Crop salt tolerance as affected by root morphology and transpiration driven salt accumulation in the rhizocylinder volume.

understanding of these processes has the potential to explain why promising results gained with various plants in soilless experiments often disappoint under soil cultivation. Water supply of shoots and water extraction by roots are decisive factors for crop salt tolerance, but can be analyzed effectively only in soil grown plants.

The sketch presented in Figure 45.7 indicates the principle contribution of root morphology and rhizospheric soil volume for salt stress alleviation. It is shown that during a period of soil water depletion, plant transpiration triggers an accumulation of salts in the root surrounding soil fraction and a salinity gradient between bulk and rhizospheric soil is built up. In case of a larger rhizocylinder volume (Figure 45.7, right-hand side), increase of rhizospheric soil solution salinity per unit of root water uptake is lower and decrease of root water uptake rate is expected to be small, which favors salt tolerance. When rhizocylinder volume is smaller, increase of soil solution salinity (per unit of root water uptake) is expected to be higher and impairs salt tolerance. In a way, enlargement of rhizospheric soil volume can be regarded as a strategy for plants to overcome osmotic stress of saline soil solutions. Hopefully, this contribution may encourage to integrate processes happening at the soil/root interface in breeding higher salt tolerance crops and optimizing brackish irrigation.

## 45.6 SUMMARY

The present concept for crop salt tolerance rating is soil based in terms of the vertical soil salinity profile under controlled irrigation. It disregards the fact that between water applications there develops a transpiration driven lateral salinity gradient between the bulk soil (soil fraction far from the root surface) and the rhizospheric soil (directly root affected soil volume close to the root surface). In field experiments in Saudi Arabia, onions were irrigated with fresh water ( $EC_i$  2.3 dS/m) and brackish waters of increasing salinities ( $EC_i$  4, 6, and 8 dS/m). Bulk soil salinity ( $EC_e$ ) increased from around 1.5 dS/m (fresh water irrigated) up to 5 dS/m (irrigated with brackish water of 6 and 8 dS/m). Rhizospheric soil salinity always was significantly higher. Immediately after a 50 mm application, rhizospheric soil was about 3–5-fold more saline than the bulk soil, but 4 days later even 6–15-fold. Plant roots differ significantly in their ability to form rhizospheric soil volumes. This feature is most relevant for the process of salt accumulation in the root surrounding soil solution and consequently for conditions of root water

uptake. Rape roots forming a large rhizospheric soil volume were in the range of 3–6-fold more effective in their water uptake from saline soils as compared to leek roots equipped with a small rhizospheric soil volume. It is recommended to include processes happening at the soil/root interface into research aiming to an enhanced salt tolerance of irrigated crops.

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# 46 Improving Crop Production on Saline Soils in Arid Regions: Do We Need a Different Approach to Develop and Select Plants for These Regions?

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## 46.1 INTRODUCTION

Salinity of the arable lands is a global problem that has restricted productivity on 955 million hectares (Mha) of lands (Szabolcs, 1987) of which 580 Mha (>60%) are sodic soils (Qadir et al., 2006). Of the 230 Mha of irrigated land, about 45 Mha (19.5%) are salt affected (FAO, 2000). The scenario is thus worse than it was 6000 years ago when the word “salinization” first appeared in the literature (Jacobsen, 1982). To date, salinity of arable lands has resulted in 26% land loss in Pakistan, 50% in Iraq, 30%–40% in Egypt, 30%–35% in Arab Republic, 27% in India, 20%–35% in the United States, 20% in Colombia, 16% in Jordan, 10%–15% in Algeria, 15% in China, 13% in Sri Lanka and Israel, and 12% in Peru (Rains, 1991). The upcoming challenge of global climate change (Guy et al., 2006) is further adding to the deteriorating situation. It is anticipated that about 30% more land loss would occur due to salinization of arable lands within the next 25 years and 50% by the year 2050 (Wang et al., 2003a). This would happen at a time when world population would be approximately 8 billion, of which 6.8 billion would be living in developing countries (Anonymous, 1998), especially in South Asia having wheat and wheat-based products as their major staple cereals. The breathtaking economic growth in developing countries of East and Southeast Asia has increased

**TABLE 46.1**  
**Global Food Distribution (kcal Person<sup>-1</sup> Day<sup>-1</sup>)**

| Region               | 1961–1969: a | 1990–1992: b | 2010: c | % Increase |       |
|----------------------|--------------|--------------|---------|------------|-------|
|                      |              |              |         | a/b        | b/c   |
| Developing countries | 1960         | 2520         | 2730    | 28.57      | 08.33 |
| Sub-Saharan Africa   | 2100         | 2040         | 2170    | −03.00     | 06.37 |
| East Asia            | 1750         | 2670         | 3040    | 52.57      | 13.86 |
| South Asia           | 2030         | 2300         | 2450    | 13.30      | 06.52 |
| Industrial countries | 3020         | 3330         | 3470    | 10.26      | 04.20 |
| World                | 2300         | 2700         | 2860    | 17.39      | 05.92 |

*Source:* Anonymous, World agriculture towards 2010, Rome, Italy, p. 83, 1998.

their purchase power, hence there is a rising trend of consuming more calories. It is anticipated that in certain countries of East Asia, intake of calories will increase by more than 14% by the end of 2010 (Table 46.1).

Having a cursory look at the current global food consumption pattern (Table 46.2), it becomes evident that consumption of calories is directly related to the status of income and inversely related to the number of undernourished people in a particular country. In order to meet the Millennium Development Goal (MDG), reducing the number of undernourished people is obligatory. Hence, it is imperative to increase the consumption of calories from 1500 to 2000 kcal person<sup>-1</sup> day<sup>-1</sup> (FAO, 2008), which requires substantial increase in calories production, a portion of which can be obtained by increasing wheat production. Also, to feed more than 6.5 billion of population by the end of 2020, the world would anyway require 1 billion metric tons (Mt) of wheat, approximately 66.5% more compared to the current production of 600 million Mt (Rajaram, 2001). Producing 400 million Mt of extra wheat would not be possible without cultivating 375 Mha of saline lands (remaining 140 Mha has already been declared as unfit for crop production), for which salt-tolerant genotypes would be required. Water availability is another problem, especially in West, Central, and South Asia, where it will drop to <1000 m<sup>3</sup> per person per annum by 2025 (Anonymous, 2000a). These stresses are among the most important reasons of persistent poverty in these areas, thereby forcing

**TABLE 46.2**  
**The World's Food Consumption Scenario (Latest as of 2008)**

| Category            | kcal Person <sup>-1</sup> Day <sup>-1</sup> | % Undernourished People |
|---------------------|---------------------------------------------|-------------------------|
| Low income          | 1500–1883                                   | >35                     |
| Lower middle income | 2000–2347                                   | 20–34 <sup>a</sup>      |
| Lower middle income | 2400–2693                                   | 20–30                   |
| Lower middle income | 2828–2880                                   | 10–20                   |
| Upper middle income | 3000–3173                                   | 05–10                   |
| High income         | 3000–3080                                   | <5                      |

*Source:* FAO, The state of food insecurity in the world. High food prices and food security-threats and opportunities, Food and Agriculture Organization of the United Nations, Rome, Italy, 2008.

*Note:* World average ranged between 2455 and 2676 kcal person<sup>-1</sup> day<sup>-1</sup>; still behind the projected average of world consumption 2860 kcal person<sup>-1</sup> day<sup>-1</sup> by 2010.

<sup>a</sup> Pakistan falls under low-income category with a consumption of 2340 kcal person<sup>-1</sup> day<sup>-1</sup> and number of undernourished people of 34%, which is now rising.

the people to migrate to other places in search of livelihood (FAO, 2005). It is also one of the reasons of prevailing malnutrition, especially in the low-income countries of Sub-Saharan Africa and South Asia (Table 46.2). The poor inhabitants of saline lands have no other means of earning and are thus migrating towards big cities creating immense burden on infrastructure and civil unrest in urban societies. Forcing them not to migrate would require huge monetary, scientific, and technical investments in order to make their lands productive. A part of this exercise relates to the availability of stress-tolerant genotypes, especially wheat which can either be selected or produced through different crop production technologies.

Concerted efforts have so far been made to develop such genotypes through various screening procedures (Kingsbury and Epstein, 1984; Rawson et al., 1988; Munns and James, 2003; Munns et al., 2006), studying (a) variability (Ashraf and McNeilly, 1988; Mass and Poss, 1989; Munns et al., 2000) and (b) mechanism of salt tolerance (Heitz and Hanson, 1980; Gorham et al., 1985; Condon et al., 2004; Munns and Tester, 2008; Mullan et al., 2009; Shavrukov et al., 2009), especially in cereals; but, except for rice (Senadhira et al., 2002), the outcome of this research has not reached the farmer fields yet. Ashraf (1994), Flowers and Yeo (1995), Munns et al. (2006) and Colmer et al. (2005, 2006) have extensively reviewed the research work conducted on salt tolerance in wheat, but the reason(s) why this research has not translated into salt-tolerant cultivar(s) and why it has not reached the farmer's field are not known. Maybe it is the extent and type of saline soils and climatic conditions that are different for different countries and require different genotypes, but again, any effort made in this direction is not adequately documented. Biotechnological approaches have also been employed (Wang et al., 2003a; Kawaura et al., 2006; Reynolds and Borlaug, 2006; Mott and Wang, 2007; Reynolds et al., 2007), but with no considerable success up to now. Is the production of only salt-tolerant plants necessary for cultivation of saline lands? Are the approaches used for tailoring salt-tolerant plants correct or a different approach needed? Can a particular wheat genotype possessing considerable levels of tolerance for salinity be cultivated on all types of saline soils or are different genotypes required? Satisfactory and logical answers to these questions are also not available in the existing literature. In this chapter, efforts have been made to describe in detail (1) the status of salinity that is coupled with desertification, high temperature, and drought-like situation, (2) type of salinity and saline soils, (3) development and status of existing salt-tolerant wheat genotypes, (4) strategies and rationale of developing new genotypes through new approach, and (5) possible impact of cultivating new stress-tolerant genotypes on saline lands in arid regions. It is hoped that the efforts would provide a new dimension and a way forward to live with salinity in a practical and productive manner.

## **46.2 MAGNITUDE AND DEVELOPMENT OF SALINITY AND DESERTIFICATION IN ARID REGIONS**

In arid regions, salinization of soil has resulted from the accumulation of water-soluble salts in soils. Very high temperature promotes evaporation and brings the salts to the surface through capillary action. The problem gets aggravated when irrigation is applied from saline tube-well water, which adds another significant amount of salts to soil surface every year (ICID, 1991). Leaching of such salts is difficult due to shortage of water and very low annual rainfall (Farooq, 2004). As a result, salinities of varying degrees have developed over large areas, of which approximately 52%, 29%, and 19% have already been classified as slight, moderate, and severely deserted, respectively (Dregne, 1986; Szabolcs, 1987). This indicates that salinity and desertification—the two very different processes—actually coexist in arid regions. It means progressive salinization either induces the development of desertification and, thus, thinning of plant cover on the soil, or it develops concurrently with desertification that reduces water availability and hinders nutrient uptake. Desertification is, however, commonly associated with salinity because it provokes salt accumulation in root zone and kills everything leaving the soil unfit for revegetation (Rozañove, 1990). In many areas, salinization and desertification alternately induce each other with disastrous consequences (Szabolcs, 1991).

Such positive correlations between the two processes (Dregne, 1986) are crucial from theoretical and practical view points (Szabolcs, 1987). The process has been observed in Turkey, Pakistan, Syria, Lebanon, and Jordan. In Arabian Peninsula, including Saudi Arabia, North and South Yemen, Oman, the United Arab Emirates, Qatar, Bahrain, and Kuwait. Extreme aridity has also promoted the development of both salinization and desertification in Egypt, Libya, Algeria, Tunisia, Morocco, and Iran, which also suffer from desertification, particularly due to salinization of irrigated lands (Rozanove, 1990). The picture is equally dark in East and South Africa, where >100 Mha of both rain-fed and irrigated croplands are affected. Desertification also prevails in India and China: China has lost 7 Mha lands due to desertification so far and its yearly growth exceeds 0.2 Mha. Salinity-induced desertification has also been reported in North, Central, and South America (Rozanove, 1990). In Australia, which is the most arid continent, salinity/sodicity prevails on approximately 250 Mha (Rengasamy and Olsson, 1991) and developed concurrently with desertification. Even in Europe, where extent and rate are the smallest, salinization and desertification have developed jointly in Spain and in Portugal (Rozanove, 1990). Salinity and desertification are, thus, among the two most common and closely related phenomena leading to deterioration of land and impoverishment of many nations, including Pakistan. Combating either of the two would (and should be) by default take into account the other. Hence, in arid regions, dealing with salinity means dealing with two divergent yet closely related processes.

### 46.3 TYPES OF SALINITY AND SALINE SOILS IN ARID REGIONS

Generally, these soils are of three types: (1) saline soils in irrigated areas, (2) saline soils in dry and/or water-deficit areas, and (3) saline soils in desert areas. The first types of soils are porous, saline and saline-sodic having electrical conductivity (EC) in the range of 10–15 dS m<sup>-1</sup> and are found saline-sodic throughout the root zone, can be easily drained, and possess good physical qualities. The extent of these soils in Pakistan is about 4.79 Mha. Approximately 1 Mha of these soils are under wheat cultivation. About 2.89 Mha out of these soils became saline due to irrigation with subsurface saline water, possess high concentration of carbonates and bicarbonates, and EC in the range of 15–20 dS m<sup>-1</sup>. Cultivation of these soils requires saline agriculture comprising salt-tolerant trees, bushes, shrubs, grasses, and plants. Soils with EC above 20 dS m<sup>-1</sup> are often known as sodic soils. The extent of these soils in Pakistan is 1.9 Mha. These are loamy, dense, and impervious to water and have high water table. Such soils also exist in Indo-Gangetic plains of India and comprise Haryana, Punjab, Uttar Pradesh, Bihar, Rajasthan, and Madhya Pradesh. Reclamation of such soils requires an engineering approach.

The second type of saline soils is generally located in water-deficit areas. Here salinity and desertification coexist. These soils are in Southern Punjab (where average temperature during wheat season ranges between 19°C and 32°C and average annual rainfall between 0.91 and 32 mm) and in some parts of Sind province (Farooq, 2004). These soils possess EC in the range of 6–10 dS m<sup>-1</sup> and exist as patches. The extent of such soils in Pakistan is 1.5 Mha and can be cultivated with high-yielding salt-tolerant genotypes that may also tolerate water deficiency/drought coupled with tolerance for high temperature. Such types of soils also exist in India, especially in Gujarat, Rajasthan, Punjab, Haryana, and Uttar Pradesh (Madan and Sharma, 1991). The causes of salinity in these soils are also similar, i.e., arid and semiarid environment, hard impervious pan in subsoil region, high water table, excessive canal irrigation, and use of subsurface saline/brackish water for irrigation. The third type of soils is peculiar to semiarid zone and includes large areas in Cholistan, Thar, Thal, and Kharan. The problems of these soils are different from irrigated areas and include high rate of evaporation and strong summer winds. Water is most critical and the limiting factor that prohibits using such areas for agricultural production. The underground water (if available) is mostly saline. In Pakistan, the extent of such soils is 11 Mha (FAO, 2000). Utilization of such soils requires genotypes specifically tailored to suit water deficiency and high-temperature conditions.

## 46.4 DEVELOPMENT AND STATUS OF EXISTING SALT TOLERANCE IN WHEAT GENOTYPES

Wheat belongs to the family Gramineae of the tribe *Triticeae*. It possesses moderate tolerance to salinity (Kingsbury, 1982). Kingsbury and Epstein (1984) tested about 6000 accessions of wheat and only 29 (0.5) emerged as salt tolerant indicating thereby that genetic base for salinity tolerance in wheat is very low and could be broadened through the use of variability available in different wheat varieties/cultivars/landraces (Torres and Bingham, 1973; Qureshi et al., 1980; Shannon, 1980; Sayed, 1985; Rashid, 1986; Mass and Poss, 1989; Munns et al., 2000; Shavrukov et al., 2009). However, in all these studies, level of variability for salt tolerance was not adequate for wheat improvement. Perennial wild wheat grasses on the other hand possess salt tolerance many folds higher than the level available in the commercial wheat varieties/cultivars, hence, these grasses have also been extensively tested and utilized.

### 46.4.1 TRANSFERRING SALT TOLERANCE FROM PERENNIAL WHEAT GRASS SPECIES

Among the perennial species, *Agropyron intermedium* and *Ag. elongatum* (syn. *Lophopyrum elongatum*; EE, syn. J\*J° or E/E;  $2n = 14$ ) are reported to be the most salt-tolerant species (Dewey, 1960). Both the genera have now been combined into one genus, i.e., *Thinopyrum* (Dewey, 1984). The tolerance of these species is associated with (1) strictly controlled influx of  $\text{Na}^+$  and  $\text{Cl}^-$  to the shoot, (2) high glycine betaine concentration, (3) reduced transpiration rate coupled with constant water use efficiency under salt stress, and (4) maintenance of high  $\text{K}^+/\text{Na}^+$  ratio in the leaves (Hitz and Hanson, 1980; Gorham et al., 1985). Since most of these studies have been conducted at seedling stage in “hydroponics”, water use efficiency (Condon et al., 2004) and carbon isotopic discrimination (Rebetzke et al., 2002)—the two most widely studied parameters under salinity and drought conditions—were largely ignored.

Efforts made to transfer salt tolerance from decaploid *E. pontica* and diploid *E. elongata* (Dvorak et al., 1985; Storey et al., 1985) were also proved successful. Amphiploids were made and tested, which indicated that several chromosomes are involved in salt tolerance (Dvorak et al., 1985), but none of these studies were confirmed in the field. A fertile plant regenerated from somatic hybrids of ultraviolet (UV)-irradiated *Th. ponticum* with protoplasts of *T. aestivum* (Xia et al., 2003) was also tested under hydroponics (Chen et al., 2004). Field evaluation of F4 and F5 generation produced grain yield equal to 4 and 6 ton  $\text{ha}^{-1}$  on saline fields indicating the introgression of genetic material from *Th. ponticum*. The wheat material, however, died before maturity and there is no report of this material being used by the farmers.

Efforts made to transfer salt tolerance from diploid *A. junceum* (*Th. bassarabicum*) to wheat (Forster and Miller, 1985; Forster et al., 1987; Mujeeb-Kazi et al., 1987, 1989) also proved successful, but the amphiploid ( $2n = 8x = 56$ ; AABBDDJJ) that survived under 250 mM NaCl solution (1) grew much faster than *Th. bessarabicum*, (2) exhibited poor fertility, (3) number of grain spike $^{-1}$  was 18, (4) grain yield plant $^{-1}$  was only 28%–33% compared to that of Chinese Spring wheat used as parental check, and (5) it was genetically unstable with 10% of the progeny not breeding true when selfed (Forster et al., 1987). The genes responsible for ion regulation in amphiploid were found on chromosomes 5J and 2J (Mahmood and Quarie, 1993; Koebner et al., 1996), which could easily be transferred to wheat but have not yet been used to develop a salt-tolerant genotype.

Genes were also transferred from diploid *Th. bassarabicum* to tetraploid wheat (King et al., 1997) and from *Lophopyrum elongatum* to hexaploid (Chinese Spring) wheat (Omielan et al., 1991). The amphiploids in both cases survived under hydroponics; however, none of these studies resulted in production of any salt-tolerant genotypes. *Th. scirpeum* was also used in crosses (Farooq et al., 1993) with hexaploid wheat. BC1 plants with various chromosomes were produced and tested. One of the plants with 44 chromosomes exhibited more vigorous growth and grain yield at EC 15 dS  $\text{m}^{-1}$  compared to 42 chromosomes lines. This line was crossed with wheat cultivar Pasban and one of the

derivatives (N-9760) appeared salt tolerant under ECe 15 dS m<sup>-1</sup> with a grain yield of 2500 kg ha<sup>-1</sup>, which indicated the salt tolerance potential of *Th. scirpeum*.

Successful efforts were also made to transfer salt tolerance from hexaploid *Th. junceum* to wheat. The disomic addition lines and partial amphiploid (Charpentier, 1992) were evaluated for salt tolerance (Wang et al., 2003b). One of the addition lines (AJDAj5) survived at EC 42 dS m<sup>-1</sup> under hydroponics and showed salt tolerance comparable to that of amphiploid. The addition line AJDAj5 retained in it a pair of chromosomes (E<sup>b</sup>E<sup>b</sup>) from *Th. junceum* (2n = 42: E<sup>b</sup>E<sup>b</sup>E<sup>c</sup>). To transfer salt tolerance from this addition line, it was crossed with hexaploid wheat carrying the *Ph*<sup>1</sup> gene. Three F5 families were selected and tested for salt tolerance of which two lines (4909 and 4910) showed salt tolerance greater than AJDAj5 and can be used as gene source for breeding salt-tolerant wheat cultivars. The two lines are still being used to assess the difference between the two through the use of micro-array analyses (Mott and Wang, 2007), but the practical outcome has not yet reached the farmer's field.

It appears that most of the studies involving salt tolerance transfer from perennial wild wheat grasses were probably made to see transfer of salt tolerance genes from wheat grasses to wheat and monitor the expression of the genes in the wheat background (Koeberner et al., 1996; Wang et al., 2003a). These short-time experimentations, mostly conducted under hydroponics, demonstrated successfully the salt tolerance potential of perennial wild wheat grass and its transfer to wheat genotypes, but they have neither been tested on saline fields where desertification also prevails in addition to high temperature and water deficiency nor under irrigated saline or coastal saline fields. Consequently, no salt-tolerant cultivar has ever been developed. Some other possibilities could be that (1) in most of these transfers, wheat cultivar Chinese Spring has been used, which is susceptible to many diseases and being used as disease spreader line in some parts of the world where it is being used as disease spreader line in disease screening nurseries (Anonymous, 1997), (2) in many cases hybrids have not been advanced beyond amphiploids that generally produced low yield under field conditions (Forster et al., 1987), (3) in cases where addition and/or substitution lines were tested they did not show required level of salt tolerance because several chromosomes were involved (Zhong and Dvorak, 1995), (4) being unstable, their tolerance was not reproducible (Gorham et al., 1986), and (5) the introgression lines produced by crossing addition lines with *Ph* mutant were either not tested in the field (Wang et al., 2003a) or contained a lot of other characters undesirable for acceptability under field conditions (Islam and Shepherd, 1991).

#### 46.4.2 TRANSFER OF SALT TOLERANCE FROM ANNUAL GOAT GRASS SPECIES

Salt tolerance in annual goat grass species of the tribe *Triticeae* such as *Aegilops* species is known since 1989 when Farooq et al. tested more than 100 different accessions of various species and found *Ae. tauschii* (syn. *Ae. squarrosa*) to be the most salt-tolerant species. *Ae. geneiculata* (syn. *Ae. ovata*), *Ae. cylindrica*, *Ae. tricuncialis*, and *Ae. bicornis* were also found salt tolerant for the first time. Among the wheat progenitors, *Ae. tauschii* (syn. *Ae. squarrosa*)—the DD genome contributor to hexaploid wheat (Kimber and Zhao, 1983)—is more tolerant (Gorham, 1990) than *Ae. speltoide*, which is a probable BB genome donor to hexaploid and tetraploid wheat (Alonso and Kimber, 1983). *Triticum urartu*, a probable AA genome contributor to hexaploid and tetraploid wheat, is more tolerant (Shavrukov et al., 2009) than tetraploid wheat and other related A-genome species (*T. monococcum* ssp. *monococcum* and *T. monococcum* ssp. *boeoticum*).

Mechanism imparting salinity tolerance in annual *Triticeae* is most often correlated with low Na<sup>+</sup> and high K<sup>+</sup> concentration in leaves (Gorham et al., 1987), and water deficiency tolerance and relative water contents of the leaves (Farooq and Azam, 2007). Durum wheat genotypes have higher accumulation of Na<sup>+</sup> and poor K<sup>+</sup>/Na<sup>+</sup> ratio in the leaves (Gorham et al., 1987) and are less tolerant than bread wheat. However, significant genetic variation does exist for Na<sup>+</sup> accumulation in durum wheat (Munns et al., 2000), which could be exploited for its improvement. Varieties of hexaploid wheat have low rate of Na<sup>+</sup> accumulation and enhanced K<sup>+</sup>/Na<sup>+</sup> discrimination—a character located



on the long arm of chromosome 4D (Gorham et al., 1987). This character is controlled by a single locus (*Kna1*) and has been linked to a molecular marker on the distal end of chromosomes 4DL (Dubcovsky et al., 1996). This locus can be easily transferred to wheat (Dvorak and Gorham, 1992), and the transfer of salt tolerance can be monitored with the help of linked marker; but, no considerable progress has yet been reported in this direction.

Unlike perennial *Triticeae*, examples of successful transfer of salt tolerance from annual *Triticeae* or *Aegilops* species are significantly low. The first successful attempt was made by transferring genes from *Ae. cylindrica* (Farooq et al., 1990a,b,c) and then from *Ae. tauschii* (Schachtman et al., 1991). The fate of the material produced by Schachtman et al. (1991) is not known, but the material produced by Farooq et al. (1992) has reached the farmer's field (Farooq and Azam, 2001).

Munns et al. (2000) tested five subspecies of *Triticum turgidum* for low  $\text{Na}^+$  and enhanced  $\text{K}^+/\text{Na}^+$  discrimination in leaves and identified a land race of durum wheat that was later used for mapping QTL using AFLP, RLFP, and microsatellite markers (Lindsay et al., 2004). After identification of one of the markers gwm312 tightly linked to low  $\text{Na}^+$ , the line was selected for breeding. Crosses made between that line and Australian durum wheat cultivar Tamaroi (with high rate of  $\text{Na}^+$  uptake) were backcrossed by Tamaroi and other Australian cultivars Wollaroi and Bellaroi. The backcross progenies are being evaluated with an indication of 10%–20% initial yield improvement on saline soils (unpublished data), but release of any variety for commercial cultivar has not yet been reported out of this research.

Accessions of *Ae. tauschii* have also been hybridized with *Triticum durum* to produce synthetic hexaploids (Schachtman et al., 1991; Mujeeb-Kazi et al., 1996). Approximately 800 such synthetic hexaploids are available at CIMMYT of which about 95 have been studied for various characteristics, including tolerance to abiotic stresses (Trethowan et al., 2000). Some of these synthetics possess low leaf  $\text{Na}^+$  concentration and enhanced  $\text{K}^+/\text{Na}^+$  ratio mostly in the range of 1–5 (Pritchard et al., 2002). Those that possess  $\text{K}^+/\text{Na}^+$  ratios above 4 could be particularly useful for transferring this character to *T. aestivum*. This material has been tested under saline fields in various countries. However, so far, none of these synthetics or their derivatives has officially or unofficially been released for commercial cultivation. The reason could be that most of these lines are (1) susceptible to diseases, (2) exhibit unacceptable plant type, (3) show poor adaptability to aridity and salinity, (4) possess poor grain quality (personal communication of the author to the breeder in AARI, Faisalabad, Pakistan), or (5) amenable to reversion (unpublished data).

#### 46.4.3 TRANSFER OF SALT TOLERANCE THROUGH GENETIC ENGINEERING APPROACHES

Biotechnological approaches such as application of molecular markers (Lindsay et al., 2004) and genetic engineering (Cushman and Bohnert, 2000) are also being used for developing salt-tolerant wheat genotypes (Chen and Murata, 2002), but nothing has been documented about field trials. The expression of HVA (LEA III protein family) in transgenic wheat improved its biomass productivity and water use efficiency (Sivamani et al., 2000). The most significant contribution is the development of salt- and drought-tolerant transgenic wheat through engineering mannitol-producing chimerical gene derived from corn and two common bacteria (Abebe et al., 2003). Transgenic wheat plants that accumulated mannitol in leaf tissues showed improved productivity under salinity and water stress. The field trials being conducted since 2004 have now been abandoned. So far, no GM wheat is being grown anywhere in the world because farmers are skeptical about the marketing of the GM crops, especially in the European countries where hesitation, fear, and confusion prevails about the future of the GM crops. Skepticism also prevails about the success of drought and salinity tolerant genotype produced through genetic engineering. In most of these plants, manipulations are based on genes protecting and maintaining the functions and structure of cellular components (Zue et al., 2004). Compared to monogenic traits, engineering genetically complex multiple genes responsible for abiotic stresses are more difficult (Wang et al., 2003a). Besides, most of these plants are largely growing in the greenhouses (Anonymous, 2004) instead of the farmer's field, and their

interaction with field environment is generally unknown. If genes involved in osmotic protection, transcription factor expression, and maintaining ion homeostasis activities are incorporated in single cultivar, they would probably work linearly to combat abiotic stresses (Pellegrineschi et al., 2005). Such a challenging task could be achieved either by transformation with multiple genes or by crossing plants containing different stress-tolerance genes. The later approach seems more feasible if the gene donor belongs to the tribe *Triticeae*; nevertheless, it has not yet been very successful. There could be many factors responsible for this bottleneck. It is now an established fact that the expression of salinity stress tolerance depends on the overall environmental factors under which the genotypes are grown (Qualset and Corke, 1991). Since annual goat grass species are known for wider adaptability (Feldman and Sear, 1981) and have already been successfully used for improvement of disease resistance in wheat cultivars, it is therefore anticipated that salt-tolerant genes derived from goat grass species will also work the same way under different saline soils and arid environments.

## 46.5 STRATEGIES AND RATIONALE OF USING NEW APPROACHES

Salinity is a complex global problem that cannot be solved by simply transferring/engineering tolerant genes into plants. Instead, its solution depends upon the gravity of the situation, priorities of the agriculture sector, resources of the countries, climatic conditions, and social acceptance of the approach in terms of economic benefits. A multidisciplinary approach is, therefore, required that can meet these challenges.

In Pakistan, for example, about 6.3 Mha of land has been affected by salinity and 11 Mha by desertification (FAO, 2000). The estimates about saline soils vary with various people and ranges between 4.22 Mha (Gassemi et al., 1995) and 6.30 Mha (Alam et al., 2001). The country's first priority in agriculture sector is increasing and achieving wheat production target of over 26 million ton by the end of the current year 2010 (Anonymous, 2000b), which means more than domestic consumption. There are about 4.1 million small farmers possessing 2.3–4.5 ha of land (their only source of livelihood) that is being degraded at a rapid pace due to salinity and drought. Presently, water is 30% less (Anonymous, 2000a) than that available previously and is further diminishing (Anonymous, 2005). Most of these areas are also beset with high temperature and heat. The farmers need new technologies and genotypes to make their lands productive with minimum possible inputs without sacrificing food and social security of their families.

To cope with this situation, it is imperative to develop wheat genotypes that can simultaneously tolerate salt and water deficiency in addition to low input requirement. This is necessary for two reasons. First, the 4.1 million small farmers do not possess enough resources to invest in their land in the form of fertilizers that are not only expensive, largely imported, but are often unavailable at crucial stages of crop growth. Second, the nitrogenous fertilizers are not only inefficient in terms of plant uptake and use [because of the low potential of soils to retain fertilizer N through immobilization as the organic matter content of soils in arid regions is low (Azam, 1983)], but also pose a threat to environmental safety and cleanliness. Hence, a multidisciplinary research program was initiated to transfer salt tolerance from wild species to commercial wheat cultivars so that resource-deficient farmers can be provided with new genotypes, new practices, and technologies that are economically viable, environment friendly, and socially acceptable. The annual goat grasses (*Aegilops* species) were selected because of several reasons (Farooq, 2004) and also because species such as *Ae. geniculata*, *Ae. umbellulata*, *Ae. speltoides*, *Ae. triuncialis*, *Ae. neglecta* and *Ae. longissima* possess considerable tolerance for drought (Rekika et al., 1997) and salinity (Farooq et al., 1989) and, thus, can be grown in water deficient/drought areas, where salinity also prevails. More importantly, *Aegilops* species are progenitor species to wheat and characterized by profuse tillering that can help produce high biomass and provide green cover to the otherwise barren land. The net result could be a reduction in desertification, amelioration of soil, and improvement in the environment (Madan and Sharma, 1991).

While viewing these characteristics, it was envisaged that (1) salt tolerance from *Aegilops* species would be transferred through introgression of genes located largely on DD genome of wheat and that of *Ae. tauschii* and/or *Ae. cylindrica*, (2) amphiploid, addition and/or substitution of chromosomes would not be required like that in case of perennial wild grasses, (3) due to wider adaptability, genotypes  $\times$  environment interaction would not be significant, (4) the derivatives of wheat  $\times$  *Aegilops* species would largely be tolerant to most of the wheat diseases and would have acceptable grain quality, (5) the drought tolerance in *Aegilops* species would induce tolerance to water deficiency in the resultant material, and (6) the ability of profuse tillering will compensate for yield losses (if any) that may occur under saline fields. All these factors were probably not considered during transferring salt tolerance from perennial wild wheat grasses.

To initiate the program, *Ae. cylindrica* ( $2n = 4x = 28$  CCDD) was used for the first time as a source of salt tolerance to improve hexaploid wheat cultivars LU-26 and Pak-81 (Farooq et al., 1992). The germplasm thus produced exhibited several folds higher tolerance than reported earlier. It survived and matured at EC 25 dS  $m^{-1}$  under hydroponic (Farooq et al., 1995) and at ECe 15–20 dS  $m^{-1}$  under field conditions (Farooq et al., 1998). Wheat lines WL-1076 and WL-41 showed significantly higher yields compared to LU-26 that was one of the parents of these lines and used as a salt tolerant check (Farooq et al., 1992). These lines require only three irrigations instead of five or six given to the commercial cultivars and half the recommended dose of both urea and phosphate fertilizers (Farooq and Azam, 2001; Farooq, 2002), which confirms the low inputs requirement of these lines.

Two of these genotypes, WL-1076 and WL-41, are being used in the country by progressive and resource-deficient farmers, especially those residing in the areas beset with water shortage. There could be several facets for acceptance of these genotypes by the farmers, but two of them, i.e., economic viability and social acceptance, are very important. The impact of economic viability can be anticipated by looking at the saving in fertilizer, irrigation water, and seed rate  $ha^{-1}$  used for cultivating saline lands (Table 46.3). It is clear that farmers are investing less and getting more income when they plant their field with stress-tolerant material. It saves 50% of water, which is a precious

**TABLE 46.3**  
**Comparison of Normal and Low-Input Agricultural Practices**  
**for Input/Output Relationships of Stress-Tolerant Wheat Lines**

| Input/Output ( $ha^{-1}$ )                               | Agricultural Practices            |                                               |
|----------------------------------------------------------|-----------------------------------|-----------------------------------------------|
|                                                          | Normal                            | Low Input                                     |
| Irrigations                                              | 5 (>Rs. 5,000)                    | 3 <sup>a</sup> (Rs. 3,000)                    |
| (1) Di-ammonium phosphate (DAP) at 957 bag <sup>-1</sup> | 5 bags (Rs. 4,785)                | 2.5 bag (Rs. 2392.5)                          |
| (2) Urea fertilizer at Rs. 527 bag <sup>-1</sup>         | 7½ bags (Rs. 3,952)               | 3½ bag (Rs. 1844.5)                           |
| (3) Seed rate <sup>b</sup> at Rs. 350/40 kg              | 200kg $ha^{-1}$ (Rs. 1,400)       | 100 kg $ha^{-1}$ (Rs. 700)                    |
| Average grain yield                                      | 4,500kg $ha^{-1}$                 | 4,500kg $ha^{-1}$                             |
| Total investment                                         | Rs. 15,137 (\$252)                | Rs. 7,937 (\$132.28)<br>(52% less investment) |
| Total income                                             | Rs. 56,250 (\$937.5) <sup>c</sup> | Rs. 56,250 (\$937.5) <sup>c</sup>             |
| Net profit                                               | Rs. 41,113 (\$685)                | Rs. 48,313 (\$805)<br>(17.51% higher profit)  |

(1), (2), and (3): Rate fixed by the government during 2006–2007.

<sup>a</sup> In case of rain, only two irrigations are provided.

<sup>b</sup> The recommended seed rate for November sowing is 150kg  $ha^{-1}$  and for January is 250kg  $ha^{-1}$ . Material produced at NIAB is always used at the rate of 100kg  $ha^{-1}$ .

<sup>c</sup> Total income is money received after selling the grains @ Rs. 500 per 40 kg. (Government rate).

commodity; hence, it is socially acceptable. It saves 50% of the money spent on the purchase of fertilizer; hence, it is economically viable.

The social acceptance of the material has been witnessed through its cultivation by the progressive farmers and cotton growers in Southern Punjab, Pakistan who like the material because of its tolerance to multiple stresses, including low light intensity and ability to grow in standing cotton crop. In fact, these farmers can continue picking cotton for extended periods after sowing of wheat in standing cotton crop. Previously, cotton was the only crop being largely cultivated in this area for two reasons. First, cotton is a cash crop and the farmers preferred to wait till the end of December to pick the last piece of the lint. Hence, the fields would not be available in November for wheat sowing. Second, in most of this area, cotton is cultivated on beds and irrigation is provided through furrows. Due to upward movement of salts through capillary action the beds are salinized. The wheat genotypes produced at NIAB being salt and water deficiency tolerant are excellent for planting on such beds. Thus, by cultivating wheat on these beds inside standing cotton (which is not a normal practice), farmers have been able to get wheat along with cotton crop, which was otherwise not possible. They have not only realized the potential of the new genotypes and technology, but also saved water and fertilizer because their selected wheat genotypes require low inputs.

After successful transfer of salt tolerance from *Ae. cylindrica*; efforts were made to transfer salt and water deficiency tolerance from *Ae. geniculata* (Farooq and Azam, 2007). *Triticum trugidum* ssp. durum was crossed as female parent with an accession of *Ae. geniculata* tolerant to salinity (Farooq and Azam, 2001) and water deficiency (Rekika et al., 1997). Consequently, "DURUGEN" was produced as a fully fertile amphiploid/allopolyploid in which doubling of chromosomes occurred automatically probably through fertilization of unreduced gametes. Field evaluation of "DURUGEN" indicated significantly low reduction in biomass and grain yield under water deficiency and salinity, respectively, compared to the reduction observed earlier in salt-tolerant wheat genotypes and cultivars grown under similar conditions. Repeated field trials of "DURUGEN," especially under artificially created desertification conditions, proved its ability to provide green cover to the barren land that reduced the dust pollution; hence, it is environment friendly. If it could not be cultivated directly, it could be used indirectly in breeding programs aiming at improving stress tolerance of cultivated wheat.

#### 46.6 POSSIBLE IMPACT OF CULTIVATING SALT-TOLERANT GENOTYPES ON SALINE SOILS

Salt-affected soils are characterized by high salt contents (sometimes high pH as well) and low content of organic matter and nitrogen (Azam, 1983). Organic matter content of these soils is low because of the inherently low vegetation-supporting potential and, thus, low quantities of plant residues reaching the soil as organic matter amendment. While halophytes can grow well under these conditions, most crop plants are highly sensitive. Several approaches have been adopted to cope with the problem of soil salinity and to use these soils for agricultural activities (Sandhu and Malik, 1975; Malik and Azam, 1979). These include (1) chemical treatment like application of gypsum and sulfuric acid, (2) leaching of salts by excessive irrigation, (3) improving surface and subsurface drainage conditions, (4) biological methods, including organic amendments, and introduction of salt-tolerant plants, and (5) finding as well as engineering plant types suited to saline environments. In most of these approaches, problems arising due to excessive salts have been given major consideration, while low organic matter content induced nutrient constraints and limited microbial activity in these soils have received comparatively little attention. Introduction of salt-tolerant crops will alleviate some of these constraints and improve the fertility and productivity of these soils mainly through Carbon (C) inputs and the consequences thereof. In addition to that, cropping salt-affected lands with newly developed salt-tolerant wheat genotypes will not only increase the overall grain production, but will have a sustainable positive effect on the physicochemical and biological properties of the soil. The effect will be realized through root activity in terms of (1) opening up

of the soil, (2) transportation of photosynthetically fixed C into the deeper soil layers, (3) rhizorespiration ( $\text{CO}_2$  released through oxidative processes of roots and rhizospheric microorganisms), (4) rhizodeposition (root exudates and sloughed off root materials), and (5) microbial synthesis of phytohormones, polysaccharides, and phenolic polymers, etc., at the expense of rhizodeposits. Each of the products of root action affects physicochemical, biochemical, and biological properties of the soil directly as well as indirectly. For example, the above-ground dry biomass of wheat produced on normal agricultural soils may vary between 10 and 20  $\text{ton ha}^{-1}$  with 40% allocation to the grain portion. Considering carbon content of the biomass to be about 35%, 3.5–7.0 ton of C is being fixed photosynthetically by wheat during about 5 months of its life cycle. It is a common knowledge that annual plants (including, wheat) grown under arable conditions transport below ground about 30%–50% of the photosynthetic C during their life cycle (Domanski et al., 2001). In fact, almost all organic C found in soil is primarily plant-derived in the form of root/shoot residues and root exudates (Kuzyakov and Domanski, 2000). An amount equivalent to 1.75–3.5 ton of C could, thus, be transported below ground. This is a substantial addition to the native organic matter content of the soil that may vary between 10 and 15  $\text{ton ha}^{-1}$  in most of the wheat growing soils. Under saline conditions, this quantity will decrease to variable degrees depending upon the extent of salinity, while rhizodeposition may increase due to salt-induced leakiness of cells (Lodhi et al., 2006). In any case, biomass yield and rhizodeposition of salt-tolerant wheat lines will be less affected by salinity. It is expected, therefore, that substantial quantities of C will be transported below ground, thereby, significantly increasing the organic matter level of the salt-affected soils. This improvement in organic matter content will be more important for the deeper soil layers that receive organic matter only through root activity and rhizodeposition. Thus, the biological activity of the deeper soil layers will also be improved.

A significant proportion of the rhizodeposits is lost through rhizospheric respiration that may represent 51%–89% of the total  $\text{CO}_2$  efflux from soil; half of this coming from root respiration (Kuzyakov et al., 1999). The release of  $\text{CO}_2$  may be of immense significance for salt-affected soils, most (if not all) of which are calcareous in nature and, hence, contain significant amounts of  $\text{CaCO}_3$ . Decomposition of  $\text{CaCO}_3$  due to the release of acidic products of organic matter decomposition together with  $\text{CO}_2$  resulting from rhizorespiration will make  $\text{Ca}^{2+}$  available to replace  $\text{Na}^+$  from the exchange complex. The effect could be supplemented through root-induced enhancement in the decomposition of soil organic matter, i.e., the so-called priming effect of root exudates (Gregory and Atwell, 1991; Kuzyakov and Domanski, 2000; Azam et al., 2004; Azam and Farooq, 2005; Kuzyakov, 2006). The role of  $\text{CO}_2$  is important not only in soil chemistry, but its elevated levels due to rhizorespiration will enhance photosynthetic activity of the crops, particularly, when they are faced with abiotic stresses like salinity and low water availability (Rozema et al., 1991; Idso and Idso, 1994; Azam and Farooq, 2001, 2003; Wall, 2001).

The rhizodeposits by plant roots generally consist of carbonaceous materials that are fairly labile and, hence, easily decomposed by soil microbes. The degradative and synthetic activities of microorganisms at the expense of these materials will lead to the production of a battery of compounds varying in complexity from  $\text{CO}_2$  to high molecular weight nonaromatic (polysaccharides) and aromatic (phenolic polymers such as humic acids) compounds. Each of these products, including humic compounds that improve root activity both under saline and nonsaline conditions (Azam and Malik, 1983; Malik and Azam, 1985), may exert a significant beneficial effect on chemistry, physics, biochemistry, and microbiology of the soil with consequent effects on plant growth as well. Similarly, bacterial exopolysaccharides synthesized in the plant rhizosphere improve the aggregation/adhesion of soil particles and, thus, the soil structure (Ashraf et al., 1999). Root-induced enhanced microbial activity may also include production of plant growth regulators, improvement in symbiotic  $\text{N}_2$  fixation, and solubilization of phosphorus, particularly, under saline/alkaline conditions (Höflich et al., 1994). Microbial population and diversity is reported to be significantly higher in the rhizospheric than non-rhizospheric soil (Germida et al., 1998; Alvey et al., 2003). Thus, growing salt-tolerant wheat will not only augment staple supplies, but will have long-term and sustainable beneficial

effect on soil characteristics (physicochemical and biological), environment (green cover), and socioeconomic status of the farming and business communities.

## 46.7 CONCLUSION

From the aforementioned text, it appeared that cultivation of saline soils is extremely important to increase productivity required to combat poverty and malnutrition. For this purpose, production of the salt-tolerant genotypes is imperative, which are in fact abundantly available, but, unfortunately have not produced the desired results. Hence, approaches used to develop such genotypes need some technical modifications making them suitable for cultivation under saline conditions coupled with desertification, water deficiency, drought, and high temperature. A genotype having tolerance for anyone of these stresses alone will probably not perform under all types of saline soils and/or saline environments. Since all these stresses have different mechanisms and set of genes controlling these mechanisms, genetic engineering approach will also not be successful. In *Triticeae*, however, huge genetic variability exists for both biotic and various abiotic stresses. Also, extensive breeding efforts made over the years to transfer salt tolerance from perennial as well as annual species like *Aegilops* have produced many different genotypes that show desired traits in regionally adapted farmer's crops. Since these genotypes are in harmony with the local environment and the stresses prevailing there, crossing these genotypes, and combining their desirable characters in one genotype for the production of a variety suitable to a particular environment, should not be difficult. Stress-tolerant varieties suitable to specific environment have been, are being, and will perhaps be produced in a similar way. This is the only way to use saline lands for increasing wheat productivity and improving its status till the time when other technologies compete with and replace these practices.

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# *Part X*

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## *Beneficial Aspects of Stress*

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# 47 Salinity-Induced Enhancement of Horticultural Crop Quality

*Catherine M. Grieve*

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## 47.1 INTRODUCTION

Salinity is the major limiting factor in crop production in irrigated agriculture and constitutes the most serious water-quality threat in many rivers and groundwater systems located in arid and semi-arid regions. The problem of salinity in agriculture is not new. Historically, societies that judiciously managed their soil and water resources survived. Other less prudent societies succeeded temporarily, but failed eventually. The rise of the Mesopotamian civilizations of the Fertile Crescent has been attributed to the development of irrigated agriculture and their subsequent decline to rising water tables and soil salinization. The ancient Sumerian farmers attempted to cope with salinization by fallowing their land and replacing relatively salt-sensitive wheat with barley, a more salt-tolerant crop. Their efforts failed and the great cities of the Tigris–Euphrates valleys disappeared only to

be replaced by vast expanses of saline marshlands or sterile desert [1–3]. The Sumerian civilization lasted for about 3000 years, longer than any other civilization to this day. In contrast, the advances we have made to manage and control salinity in arid regions of the world are still in their infancy. The Bureau of Reclamation was founded only a century ago; California's Central Valley Project was built less than six decades ago [2]. Nevertheless, the seriousness of the problem has been recognized, and sustained efforts continue to be directed toward heading off the disastrous effects of salinity [1,2].

Most arid or semiarid areas of modern irrigated agriculture are subjected to growing water security uncertainties. Surface and subsurface water allocation is a complex system of competing and interacting demands from agricultural, urban, industrial, environmental, tribal, and recreational groups. Agricultural water users, faced with less water or with lower-quality water than desired, have responded by developing improved and innovative soil, water, and drainage management strategies in order to effectively use alternative water resources: urban wastewaters, recycled drainage waters, seawater-contaminated well waters, and other low-quality waters. Such strategies will conserve fresh water supplies, reduce the volumes of drainage waters requiring disposal, minimize discharge of salts and potentially toxic trace elements to the environment, reduce the area affected by shallow water tables, and optimize land productivity.

Improved management practices for crop production under irrigation with brackish waters have been developed to minimize yield losses and other detrimental effects due to salinity stress. Over the years, it has become increasingly obvious to growers and researchers alike that the controlled reuse of alternative saline waters not only improves environmental stewardship, but also offers a second benefit by providing a unique opportunity for producing value-added crops [4–6]. Quality of fruit and vegetable crops, medicinal crops, and ornamental crops can be improved by judicious management of saline irrigation waters. The purpose of this chapter is to illustrate the beneficial effects of salinity on selected plant species and to highlight research directed toward minimizing yield losses and maximizing quality of salt-stressed crops.

## 47.2 PLANT RESPONSE TO SALINITY

The salt tolerance of a crop is best described as a complex function of yield decline across a range of salt concentrations [7–10]. Maas and Hoffman [10] proposed that the response curve could be represented by two line segments: (1) a tolerance plateau with zero slope, representing the maximum soil salinity that does not reduce yield below that obtained under nonsaline conditions; and (2) a concentration-dependent line whose slope indicates the expected yield reduction per unit increase in salinity. The “threshold-slope model” has been used to compile data for 141 crop species that provide information for growers concerning potential salinity hazards to crops [11]. Model parameters are given in terms of the electrical conductivity of the saturated soil extract ( $EC_e$ ), the traditional soil salinity measurement with units of deciSiemens per meter ( $dS\ m^{-1}$ ) [11]. As an approximation,  $EC_e = 1.5$  times the electrical conductivity of the irrigation water ( $EC_{iw}$ ) assuming steady-state conditions and a leaching fraction between 15% and 20% [12].

The initial influence of salinity stress on plants is growth suppression, a typically nonspecific salt effect that depends more on osmotic stress created by the total concentration of soluble salts in the rootzone than on the level of specific solutes [13]. The rapid osmotic phase starts immediately after salt accumulation in the rootzone exceeds some threshold level. Subtle changes then occur due to water loss from leaf cells and reduction of cellular volumes. Within hours, however, the plant adjusts osmotically, cell volumes and turgor are restored, but the irreversible damage has already been done. Cell elongation and cell division are reduced, and, as a result, shoot growth decreases, leaves expand and emerge more slowly, and fewer lateral branches form [14]. Roots are also reduced in length and mass. Moderate salinity levels usually restrict growth without any overt injury symptoms and the plants appear normal, but stunted. Compared to non-stressed leaves, salt-affected leaves are smaller and thicker; their color may be darker blue-green due to increased chloroplast density per unit leaf area, and their surface may be coated with a pronounced waxy bloom [15].

The survival of a salt-stressed plant depends primarily on its capacity to acquire essential mineral nutrients from its root environment and to synthesize bioactive compounds in order to extract water from the root media and regulate turgor potential. Depending upon the degree of stress, these mineral ions and organic compounds may contribute to osmotic adjustment (OA) and assure that the plant is healthy enough to complete its life cycle. The presence of such constituents generally improves crop quality and demonstrates that not all aspects of salinity are negative. When fruits and vegetables from salt-stressed plants become part of the human diet, they may provide desirable health benefits beyond basic nutrition. Two classes of phytochemicals contribute to improved quality: compatible osmolytes and antioxidants.

#### 47.2.1 COMPATIBLE OSMOLYTES

Plants are able to adapt to and survive in saline environments by the net accumulation of solutes to maintain a favorable water potential gradient. The concept of osmoregulation and the development of the intercellular model of solute compartmentation is due largely to the pioneering work of John Gorham [16], Gareth Wyn Jones [17,18], Timothy Flowers [19], and their research teams. The principal features of the model are as follows: (1) under saline conditions, the large quantities of salts that are transported to the leaves and contribute to OA are accumulated mainly in the vacuole where tissue concentrations can exceed  $200 \text{ mol m}^{-3}$ ; (2) the concentration of inorganic ions in the cytoplasm is held in the range  $100\text{--}200 \text{ mol m}^{-3}$  and the cytoplasm shows a strong selectivity for potassium over sodium, magnesium over calcium, and phosphate over chloride or nitrate; (3) the maintenance of osmotic equilibrium across the tonoplast requires the accumulation in the cytoplasm of nontoxic organic solutes [16]. Thus, osmotic balance is achieved by the preferential compartmentalization of the mineral ions in the vacuoles and the compatible solutes in extravacuolar compartments. More recently, Wyn Jones and Gorham [20] revisited the basic features of the solute compartmentation model, and now caution that it is totally misleading to treat vacuoles as inert balloons inasmuch as there are at least two types of vacuoles, one with high salt concentrations and another with high organic solute concentrations.

OA can be accomplished with relatively inexpensive energy expenditure by uptake and transport of inorganic ions, such as  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{NO}_3^-$ , or  $\text{Cl}^-$ . The more energy-demanding feature of OA is the biosynthesis and accumulation of low-molecular-weight organic compounds, a strategy that occurs at the expense of biomass production [21]. The organic osmolytes or “compatible solutes” generally found in higher plants include sugars (mainly sucrose and fructose), sugar alcohols (methylated inositols), complex sugars (trehalose, raffinose), soluble proteins, amino acids, quaternary ammonium compounds (betaines, choline *O*-sulfates), polyamines, and sulfonium compounds [22–24]. Osmobalancers may accumulate to high levels under osmotic stress without disturbing the proper functioning of organelles. Compatible solutes generally have a primary role in turgor maintenance and may also be involved in stabilizing proteins and cell structures [23,25]. However, the main contribution of osmolytes may not be to OA, but rather, their metabolic pathways may have adaptive value [22] or, alternatively, osmolytes may be involved in other counteracting functions that remain to be investigated [23].

#### 47.2.2 ANTIOXIDANTS

Reactive oxygen species (ROS) are free radicals, continuously produced in plants during normal metabolic processes such as photosynthesis and respiration [14,26]. Different forms of ROS are produced including superoxide ( $\text{O}_2^-$ ), hydrogen peroxide, and the hydroxyl radical ( $\text{OH}^\bullet$ ). In addition, the triplet state of chlorophyll can interact directly with ground state triplet oxygen, returning the chlorophyll molecule to its singlet ground state and forming highly reactive singlet oxygen [27]. These activated oxygen species are cytotoxic, and their rapid and nonspecific reactions can disrupt normal metabolism through oxidative damage to all classes of biomolecules, including lipids, proteins, chlorophyll, and nucleic acids [28]. Plants have the ability to scavenge and/or detoxify ROS, however, by the production

of two classes of low-molecular-weight antioxidants, i.e., hydrophilic (present in the aqueous phase) and hydrophobic (present in cellular membranes). The hydrophilic antioxidants include ascorbate, cysteine, flavonoids, coumarins, and polyphenols. The hydrophobic antioxidants include tocopherols (vitamin E), carotenoids, xanthophylls, flavonoids, and vitamin D. Enzymatic antioxidants, such as superoxide dismutase, catalase, and a variety of ascorbate peroxidases are capable of regulating ROS levels [29,30].

Production of ROS in the cell is very low under normal growth conditions, and these species are involved in plant development as signals for growth, movement, and differentiation, as well as for acclimatization to changing environmental conditions [27]. However, environmental and biological factors, such as pathogens, aging of plant organs, hyperoxia, light, drought, pollutants, and salinity are known to enhance ROS production to such a degree that the defensive antioxidant system capacity is overcome, triggering oxidative stress and causing membrane lipid peroxidation, enhanced membrane leakage, and tissue deterioration. Salt stress, for example, enhanced  $O_2^-$  production by submitochondrial particles and lipid peroxidation of mitochondrial membranes in NaCl-sensitive pea plants [30]. The coordinated activity of the enzymatic and nonenzymatic antioxidants achieves a balance between the rate of formation and the rate of detoxification of ROS [27,30].

The biochemical and molecular mechanisms that plants have developed to cope with salinity stress result in the acquisition and formation of components, many of which are essential to human health. The compatible solutes that accumulate in response to salinity enrich plant foods in essential mineral nutrients, carbohydrates, lipids, and proteins [31]. Humans are not able to synthesize many of the antioxidant molecules present in plants, but are apparently able to absorb and utilize these phytochemicals to limit oxidative damage caused by normal metabolic processes as well as from external sources that may cause tissue damage. The ability of humans to harness plant-derived antioxidant defenses may reduce the risk of age-related degenerative diseases, including heart disease, cataract formation, and cancer [27,31–33]. Antioxidants include vitamins E and C,  $\beta$ -carotene, some trace elements that are components of antioxidant enzymes (including selenium, copper, zinc and manganese), and phenolic compounds (including phytoestrogens, flavonoids, phenolic acids). The concentrations and profiles of phytochemicals in fruits and vegetables depend on genetic and environmental factors (temperature, light intensity, salinity, water availability, soil type) [34,35], and on cultural practices (species and variety selection, fertilization practices, planting density, irrigation method, date of harvest) [36]. When challenged by salt stress, plants produce a wide variety of these phytochemicals, and while not all of these constituents are strictly required in human diets, they promote good health when present in sufficient levels [37,38]. There is strong evidence that a diet rich in naturally occurring phytochemicals present in fruits and vegetables is more effective than the same phytochemicals consumed as the purified products or extracts [39,40].

### 47.3 SALINITY EFFECTS ON QUALITY OF FRUITS AND VEGETABLES

Improvement of crop quality by addition of salts to plant rooting media is not new. Tottingham [41] noted in 1919 that carrot and radish fertilized with NaCl were higher in sugar content and more flavorful than those in the nonsaline control plots. Increased flavor was not surprising as chefs have long known that sugars together with inorganic salts (NaCl, KCl, and others) improve the taste and flavor balance of foods [42]. As methodology for analyzing plant constituents developed, quality variables of fruits and vegetables were reported based on total dissolved solids (TSS), sugars, and titratable acids (TA). Further advances in technology and development of innovative instrumentation permitted the routine analysis and quantification of compatible solutes [43,44], antioxidants [45,46], and other health-promoting phytochemicals.

Numerous strategies have been tested for minimizing crop yield loss due to salinity, and at the same time, maximizing inner (nutrient value, taste, texture) and outer (appearance, color, firmness, shelf life, aroma) quality characteristics of the marketable product. Those management practices are presented below.



### 47.3.1 NUTRIENT MANAGEMENT OF SALT-STRESSED CROPS

Low to moderate levels of NaCl are often used to improve fruit quality. The practice of using NaCl as the sole salinizing agent may, however, lead to injury in crops that are susceptible to Na<sup>+</sup> and Cl<sup>-</sup> toxicity [47] and to nutrient disorders resulting from ion imbalances and interactions. NaCl-salinity, for example, may cause decreased uptake of calcium by the roots and increased resistance to its transport. Increased incidence of disorders related to calcium deficiency are common in the fruit and developing leaves on NaCl-stressed crops, e.g., blossom-end rot (BER) of tomato and pepper, internal fruit rot of eggplant, bud rot of artichoke, blackheart of celery, and internal tip burn of Chinese cabbage. Potassium acquisition and uptake may be inhibited by high substrate-Na which, in turn, influences K<sup>+</sup>/Na<sup>+</sup> by shifting the uptake ratio in favor of Na<sup>+</sup> at the expense of K<sup>+</sup>. In addition, crop N status may be impaired by high external Cl<sup>-</sup> [48]. Many research teams acknowledge that mineral nutrient problems can arise when NaCl is the only salinizing salt, but continue to use the NaCl system because the results seem to be simpler to interpret as compared to the complex solution chemistry inherent in the use of alternative water resources, e.g., drainage effluents, seawater-contaminated well waters, and nursery run-off waters. As a consequence, considerable research has been directed toward improved fertilizer practices in order to alleviate mineral ion imbalances, interactions, toxicities, or deficiencies caused by NaCl-dominated irrigation waters.

### 47.3.2 TIMING OF SALINITY APPLICATION OR WITHDRAWAL

Growth suppression by salinity can be a useful and controllable management strategy to regulate crop productivity and fruit quality, particularly in herbaceous crops that exhibit strong competition for photosynthates between vegetative and reproductive organs. Application of salinity early in the life cycle slows vegetative growth. If the stress is subsequently reduced or withdrawn entirely, excess photosynthates are released to flow to the generative parts, producing more fruit or larger fruit, often with minimal adverse effects on fruit yield. Application of salinity at a specific stage of reproductive growth may improve the quality of fruits by reducing water content, increasing the dry matter content, and increasing constituents that contribute to taste, aroma, appearance, and improved nutrition [6].

### 47.3.3 IRRIGATION MANAGEMENT: METHOD OF APPLICATION, SCHEDULING

Growers and researchers have sought to develop strategies to manage saline irrigation waters that maintain yields, but impose a moderate, controlled level of stress on the crop to improve the quality of the marketable product. Irrigation practices such as increasing the periods between irrigations or by withholding irrigations during a defined period of the crop growth cycle can constitute a sustainable tool for using available non-potable water resources more efficiently [49]. In general, increasing the water supply increased fruit yield, but reduced fruit quality due to high fruit water content. Specifically, low water tension decreased the vitamin C content of the fruit. Drip irrigation increased vitamin C content compared to surface irrigation, and vitamin C content was higher when plants are subsurface drip irrigated, compared to surface or conventional drip-irrigation systems [50].

### 47.3.4 GRAFTING: CHOICE OF ROOTSTOCKS

Grafting has been widely used to limit the deleterious effects of abiotic stresses: inducing resistance to low and high temperatures, correcting ion imbalances, excluding toxic ions, improving nutrient uptake, and enhancing water uptake. Improvement in fruit quality appears to be both rootstock- and scion-dependent with both sections contributing their own unique traits for coping with osmotic stress [51–53].

### 47.3.5 BREEDING PROGRAMS

Accumulation of compatible solutes and antioxidants in many plants has been positively correlated with salinity tolerance. Numerous studies have been initiated over the last few decades to improve plant salt tolerance using these biochemical indicators as markers [21,29]. At the same time, the health-promoting properties of compatible solutes and antioxidants have motivated several laboratories to institute genetic engineering programs designed to increase the content of the beneficial components in fruits and vegetables [54–56]. Despite the existing and potential value of agricultural biotechnology, there remains considerable consumer resistance to the acceptance of genetically modified foods [37,57]. As an alternative to bioengineering, the application of an abiotic stress, such as salinity, can serve to enhance concentrations of desirable phytochemicals in fruits and vegetables. High quality crops produced by a strictly agronomic management practice may be more acceptable to consumers.

Davis et al. [58] examined changes in nutrient content data for 43 garden food crops from 1950 to 1999. As a group, the crops declined in some mineral nutrients and phytochemical constituents, suggesting that during that period, cultivars were selected for yield, growth rate, pest resistance, and other characteristics, rather than for nutrient content. In tomato, for example, large trade-offs were found between yield and ascorbate concentration and between fruit size and ascorbate concentration. Driven by the expectations of a more health-conscious public, the goals of conventional selection and breeding programs have changed dramatically over the past decade and are now focused on developing more flavorful, nutritious food crops, while maintaining the grower-focused traits such as high yield and disease resistance [36].

The remainder of this chapter presents examples of the beneficial effects of salinity on selected crops of certain plant families, and in some cases, the experimental means by which the benefits were achieved. Salt tolerance ratings and tolerance parameters are given when available. Tolerance threshold is expressed as  $EC_e$  (T), and the slope (S), as percentage of yield reduction for each unit, added salinity above the threshold value [10,11].

## 47.4 EXAMPLES OF THE BENEFICIAL EFFECTS OF SALINITY ON SELECTED CROPS OF CERTAIN PLANT FAMILIES

### 47.4.1 AMARYLLIDACEAE

Onion (*Allium cepa* L.). Moderately sensitive.  $T = 1.2 \text{ dS m}^{-1}$ ,  $S = 16\%$  [11]. Onion is highly valued for its characteristic taste and flavor. Salinity increased sugar content [59] in the expressed juice, and the response appeared to be cultivar dependent. Sucrose concentration in the expressed juice of “Texas Early Grano” increased 45% as soil salinity increased from 0.86 to  $3.8 \text{ dS m}^{-1}$ . At the same time, however, weight of the cured bulbs decreased nearly 40% [59]. Chang and Randle [60] reported that the flavor precursors in onion (S-alk(en)yl cysteine sulfoxides (ACSOs)) increased only when external NaCl increased from 75 to 100 mM, a stress factor that reduced bulb yield by 70%.

### 47.4.2 APIACEAE

Carrot (*Daucus carota* L.). Salt sensitive.  $T = 1.0 \text{ dS m}^{-1}$ ,  $S = 33\%$  [11]. Flavor enhancement of salt-stressed carrot due to increases in sugar content was initially documented by Tottingham [41]. Quality improvement, however, occurred at the expense of crop yield. Depending on cultivar, sucrose in the storage root increased nearly 40% as soil salinity ( $EC_e$ ) rose from 0.6 to  $5 \text{ dS m}^{-1}$ , while yield fell 50% [61,62].

### 47.4.3 ASTERACEAE

Lettuce (*Lactuca sativa* L.). Moderately sensitive (iceberg type,  $T = 1.3 \text{ dS m}^{-1}$ ;  $S = 13\%$ ) [11]. Lettuce is an important source of dietary antioxidants (phenolic compounds, vitamins C and E). Mild environmental stresses (e.g., heat shock, chilling, high light intensity) improve the phytochemical content

with little or no adverse effect on lettuce growth [63]. Results of a field study in Israel indicated that the yield and quality of iceberg lettuce were not affected by sprinkling with saline irrigation waters as high as  $4.4 \text{ dS m}^{-1}$ . Romaine lettuce types tested in the study were significantly more tolerant than iceberg types [64]. Kim et al. [65] reported that, with suitable crop management practices, increases in phytochemical content can be attained without causing a tradeoff in yield or visual quality. For example, relatively mild salinity ( $\text{NaCl} = 5 \text{ mM}$ ) applied 15 days prior to harvest had no significant effect on growth or color of romaine lettuce. Saline waters with  $\text{NaCl}$  concentrations as high as  $1000 \text{ mM}$   $\text{NaCl}$  also had no effect on shoot dry weight if exposure time was short (2 days prior to harvest). Major carotenoids, lutein, and  $\beta$ -carotene in romaine increased with salt stress, regardless of timing of application.

#### 47.4.4 BRASSICACEAE

Broccoli (*Brassica oleracea* L., Botrytis group). Moderately salt tolerant.  $T = 1.3 \text{ dS m}^{-1}$ ,  $S = 15.8\%$  [66]. Cruciferous vegetables, such as broccoli, are widely recommended as nutritionally beneficial vegetables. Broccoli, in particular, is regarded as the best source of glucosinolates, a class of sulfur compounds partly responsible for the characteristic taste of members of the cabbage family. In recent years, considerable attention has been directed toward the results of epidemiological studies that associate high consumption of *Brassica* vegetables with chemoprevention of degenerative diseases and certain types of cancer [67]. Glucosinolates and their degradation products may limit cancer cell development by regulating target enzymes and controlling apoptosis [68–71]. Timing of salt stress application dramatically affected broccoli growth, productivity, and phytochemical profiles. Nutrient-complete irrigation waters amended with either 0 or  $40 \text{ mM}$   $\text{NaCl}$  were applied to “Marathon” broccoli at the head development stage (~95 days old). Biomass of the heads of salinized plants was 12% higher than that of the control plants. Moderate salinity affected phytochemical content of the heads; as salinity increased, total flavonoids increased 25% and total phenols, 20%. However, the glucosinolates and ascorbic acid were unaffected by salt treatment [68]. Subsequently, the same research team [72] reported that the application of constant salinity stress ( $40 \text{ mM}$   $\text{NaCl}$ ) from the seedling stage to maturity reduced “Marathon” head weight 76% compared to the nonsaline control heads. Relatively long-term salt stress also altered phytochemical profiles, compared to those observed when salinity was applied late in the growth cycle. The concentration of total glucosinolates in the florets of broccoli increased sixfold as external  $\text{NaCl}$  increased to  $40 \text{ mM}$ . Of the total phenolic compounds (chlorogenic and sinapic acid derivatives and flavonoids), only sinapic acid in the inflorescences increased in response to moderate salinity. The concentrations of other phenolics were reduced and vitamin C was unaffected by increasing salinity.

Cabbage (*B. oleracea* L. Capitata Group). Moderately sensitive.  $T = 1.8 \text{ dS m}^{-1}$ ,  $S = 9.7\%$  [11]. Under salt stress, cabbage heads are generally more compact and leaves are fleshier than under nonsaline conditions [62].

#### 47.4.5 CUCURBITACEAE

Melon (*Cucumis melo* L. Reticulatus Group). Moderately sensitive.  $T = 1 \text{ dS m}^{-1}$ ,  $S = 8.4\%$  [8]. Salinity improves melon fruit quality by increasing dry matter, total sugars, TA, total soluble solids [73–75], and pulp firmness [76]. Several research teams have reported substantial benefits in yield and quality of melons irrigated with low-quality waters by developing better strategies to manage the time and duration of brackish water application [74,76,77]. Bustan et al. [77] applied saline waters ( $\text{EC}_{\text{iw}} = 7 \text{ dS m}^{-1}$ ) to field-grown melons at three developmental stages: branching, appearance of first pistillate flower, and 50% peel reticulation. Differences in total yield of the plants irrigated with fresh and brackish water were small based on export quality characteristics, i.e., suitable fruit size and appearance. Fruit from plants irrigated with fresh water ( $\text{EC}_{\text{iw}} = 1.2 \text{ dS m}^{-1}$ ) were either too large, poorly netted, soft, or irregularly shaped. Botia et al. [78] studied the response

of two field-grown melon cultivars to fresh and saline irrigation waters applied at different stages of growth: (1) application of fresh water ( $EC_{iw} = 1.3 \text{ dS m}^{-1}$ ) transplant to harvest; (2) application of saline water ( $EC_{iw} = 6.1 \text{ dS m}^{-1}$ ) transplant to harvest; (3) application of fresh water from transplant to start of flowering, then application of saline water to harvest; and (4) application of fresh water from transplant to the start of fruit set, then application of saline water to harvest. Application of saline water from fruiting to harvest did not reduce marketable yield in either cultivar. Fruit quality (TSS and maturity index) of both cultivars increased. A stage-of-growth experiment was conducted under soil-less conditions by del Amor et al. [79]. Four levels of NaCl-salinity (2, 4, 6, 8  $\text{dS m}^{-1}$ ) were applied to greenhouse melons at four different times: early vegetative growth (14 days after transplant), beginning of flowering, beginning of fruit set, and beginning of fruit ripening. Yield progressively increased when salinity was imposed at later dates. Salinity treatments increased fruit reducing sugars, acidity, and TSS. These investigators also point out that the relatively high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the fruit undoubtedly contributed to enhanced flavor as suggested by Gillette [42].

Grafting is an effective tool for increasing fruit yield and quality of cucurbits [52]. Melon plants either grown on their own roots or grafted onto a commercial rootstock (*C. maxima*  $\times$  *C. moschata*) were grown in a greenhouse and subjected to a range of NaCl-salinity treatments ( $EC_{iw} = 2\text{--}9.7 \text{ dS m}^{-1}$ ) for two successive cropping years. Averaged across years and salinity treatments, marketable fruit yield of the grafted plants was 44% higher than that of the ungrafted plants. Salinity improved fruit quality of ungrafted plants through increased acidity and TSS to a greater extent than the grafted plants. Fruit from grafted plants, however, were firmer with a superior pulp color index [80].

Cucumber (*Cucumis sativus* L.). Moderately sensitive.  $T = 2.5 \text{ dS m}^{-1}$ ;  $S = 13\%$  [11]. Salinity enhanced cucumber quality by improving TSS content, shelf life, taste, and color index [81–83]. Grafting cucumber on two different salt-tolerant rootstocks resulted in improved quality through increases in vitamin C and titratable acidity. Salinity, however, caused substantial marketable yield losses of both grafted and ungrafted plants, ranging from ~50% when the plants were irrigated with saline waters ( $\text{NaCl} = 30 \text{ mM}$ ) to 75% when the irrigation waters contained 60  $\text{mM NaCl}$  [84].

Squash (*Cucurbita pepo* L.). Zucchini squash (*C. pepo* var. *melo pepo* L. Alef.). Moderately salt tolerant.  $T = 4.7 \text{ dS m}^{-1}$ ;  $S = 7.6\%$ . [11]. Salinity improves zucchini fruit quality through enhancement of physical (fruit firmness) and chemical parameters (TA, total soluble solids) [85]. Rouphael et al. [86] compared crop response to application of saline waters under greenhouse conditions by two different methods: drip and subsurface irrigation. Regardless of irrigation water salinity level (2.0 vs. 4.1  $\text{dS m}^{-1}$ ), marketable yield was always lower when the plants were irrigated by subirrigation rather than with the drip system. Increasing salinity from 2 to 4  $\text{dS m}^{-1}$  improved fruit quality (dry matter, glucose, fructose, starch, and total carbohydrate content) in fruit produced by both irrigation systems. The investigators suggest that using drip irrigation with saline waters (4.1  $\text{dS m}^{-1}$ ) would be a good management practice for limiting yield reduction while taking advantage of the salinity-induced improvement in quality.

Watermelon (*Citrullus lanatus* (Thunb.) Matsum and Nakai). Little information is available on the salt tolerance of watermelon, although it is thought to be moderately sensitive to salinity [11]. Lei et al. [87] demonstrated to local farmers in the Hetao region of Inner Mongolia that the use of saline groundwaters fluctuating from 3.3 to 6.3  $\text{dS m}^{-1}$  over the growing season was feasible for watermelon production. Three irrigation treatments were used: 30%, 60%, and 90% of Chinese pan evaporation. The traditional production method (i.e., no in-season irrigation) was used as the control treatment. Compared to the dryland cropping system, both yield and quality of watermelon increased as a result of improved irrigation management practices. Quality of the irrigated fruit was enhanced through increases in total sugars, organic acids, and vitamin C.

Watermelon grafted on commercial salt-tolerant rootstocks, e.g., *Lagenaria siceraria* or the hybrid, *Cucurbita maxima*  $\times$  *C. moschata*, showed increased yield, vigor, and quality compared to the yield of ungrafted or watermelon–watermelon grafted plants [51,52]. As irrigation water salinity rose from 2 to 5.2  $\text{dS m}^{-1}$ , fruit quality of plants grafted on salt-tolerant rootstocks improved, due

largely to increases in dry matter percentage, glucose, fructose, sucrose, and total soluble solids as well as to decreases in the percentage of seeds [51].

#### 47.4.6 LILIACEAE

Asparagus (*Asparagus officinalis* L.). Salt tolerant.  $T = 4.1 \text{ dS m}^{-1}$ ,  $S = 2\%$ . [88]. Asparagus rates as the most salt-tolerant traditional vegetable crop, and provides one of the best examples of balance in yield and quality. Soil salinity of  $10 \text{ dS m}^{-1}$  reduced spear yield 10% and at the same time increased quality in terms of total soluble solids 10%. As soil salinity increased from 2.4 to  $14.8 \text{ dS m}^{-1}$ , TSS increased 12%, but yield declined 20% [88].

#### 47.4.7 ROSACEAE

Pear (*Pyrus communis* L.). Salt sensitive [11]. Oron et al. [89] compared the response of pear to irrigation water qualities ( $1.2$  and  $4.4 \text{ dS m}^{-1}$ ) and to location of drip-irrigation emitters (surface, subsurface at a depth of 30 cm, subsurface at a depth of 60 cm). Results of this field trial indicate that the crop performed better under the conventional surface drip system in terms of yield (9%–11% for freshwater and 36%–40% for saline water irrigation) in comparison to the yields obtained with subsurface drip irrigation. Salinity applied at the 30 cm depth, however, tended to improve overall fruit quality through increased sugar content, total acidity, firmness, and extended shelf life.

Raspberry (*Rubus idaeus* L.). No available salt tolerance parameters. Neocleous and Vasilakakis [90] demonstrated that the use of poor-quality water during a portion of the cropping season increased the quality of raspberries. Saline treatments were applied to plants for 10 days commencing at the pink fruit stage using NaCl-dominated irrigation waters with ECs ranging from 1.6 to  $6.9 \text{ dS m}^{-1}$ . The application period was followed by withdrawal of stress for another 10 days. Berry dry weight increased with salinity during the application period, but was reduced to that of the non-salinized berries during the stress-relief period. Concentrations of ascorbic acid, soluble solids, titratable acidity, total phenolics, and antioxidant capacity in the berries also increased with salinity, and remained high even when stress was withdrawn.

Strawberry (*Fragaria × ananassa* Duch.). Salt sensitive.  $T = 1.0 \text{ dS m}^{-1}$ ,  $S = 33\%$  [91]. Salinity levels of  $2\text{--}3 \text{ dS m}^{-1}$  in the soil extract improved fruit quality of field-grown strawberry cultivars “Lassen” and “Shasta” through increases in TA and total sugars, a result that occurred primarily by a reduction in fruit moisture content. Total yields were reduced 50% when soil salinity was  $2.3 \text{ dS m}^{-1}$  for “Shasta” and  $2.6 \text{ dS m}^{-1}$  for “Lassen.” Quality components were influenced by local weather conditions around the time of harvest; acids and sugars were lower when fruit sampling was preceded by a few cloudy days [91].

Fruiting in strawberry is known to be antagonistic to vegetative growth, and conversely, vegetative growth beyond some critical point is detrimental to fruit yield. The controlled use of salinity to balance vegetative and reproductive growth can be a strategy for producing better quality fruit while minimizing adverse effects on yield. When application of NaCl-salinity was delayed until the 12-leaf stage and remained constant throughout the cropping season, the concentration of dry matter, reducing sugars, and sugar:acid ratios in fresh fruit increased. Improvement of strawberry quality by salinity occurred largely through a reduction of the fruit moisture [92,93]. Sarooshi and Cresswell [94] also addressed strawberry response to fluctuations in NaCl-salinity. When saline treatments were applied during early seedling growth, and withdrawn at early fruit set, the berries were heavier and sweeter and the aroma improved.

Strawberry appearance, texture, and firmness are as important as the phytochemical composition in determining quality and commercial acceptance of strawberries [93]. Quality of relatively more salt-tolerant cultivars are generally better than those of more sensitive cultivars. Keutgen and Keutgen [95] found that taste, aroma, TA, and texture decreased significantly as NaCl-salinity increased, although reduction was more dramatic for the most salt-sensitive cultivar [96].

In subsequent trials with the same strawberry genotypes, Keutgen and Pawelzik [97,98] reported that salt stress increased the antioxidant activity and antioxidant pools (superoxide dismutase, ascorbic acid, anthocyanins) as well as the concentrations of free essential amino acids (threonine, valine, isoleucine, leucine, and phenylalanine). Taste panels rated the quality of fruit from the more salt-sensitive plants as poor primarily due their softness as well as to the low content of sugars, organic acids, and soluble solids [99]. In contrast, fruit quality of the relatively more salt-tolerant cultivar was rated as superior by the panel, particularly with respect to texture and taste-relevant components [100].

#### 47.4.8 RUTACEAE

Citrus species. Salinity effects in citrus have been reviewed extensively by Levy and Syvertsen [101]. Benefits of moderate salinity are improvement of cold hardiness, enhancement of flowering if stress is applied/withdrawn at the appropriate stage of growth, and increases in juice solids and acidity. Citrus fruits contain phytochemicals such as carotenoids, limonoids, flavonones, folate, and vitamin C. The reviewers [101] speculate that controlled salinity might enhance the concentrations of phytochemicals in citrus juice, but know of no information to support that speculation. More recent studies confirm salinity-induced increases in TSS and acidity [102–104], but this effect appears to depend on the age of the trees. From the results of a long-term field study designed to quantify the effects of NaCl-salinity on yield and fruit quality of Valencia orange trees, Grieve et al. [105] reported that soluble solids were initially higher in the salinized plots, but by year 9, TSS were lower than in the control plots. Juice acidity was also initially higher in fruit from the salt-stressed trees, but decreased over time.

#### 47.4.9 SOLANACEAE

Eggplant (*Solanum melongena* L.). Moderately Sensitive.  $T = 1.1 \text{ dS m}^{-1}$ ,  $S = 6.9\%$  [106]. Sifola et al. [107] evaluated yield and quality of eggplant cv. “Mirabelle” exposed to the combined effects of NaCl-salinity ( $EC_{iw} = 2.3, 4.4, 8.5$  and  $15.7 \text{ dS m}^{-1}$ ) and irrigation frequency (every 2, 5, or 10 days). Marketable yield was significantly reduced only when salinity exceeded  $8.5 \text{ dS m}^{-1}$ . Irrigation frequency had little effect on eggplant yield or quality. However, quality (titratable acidity, reducing sugars) increased with salinity.

Pepper (*Capsicum annuum* L.) Moderately Sensitive.  $T = 1.5 \text{ dS m}^{-1}$ ,  $S = 14\%$  [11]. Pepper fruit can be consumed at different maturity stages: green, not-quite-ripe or turning, and red. Phytochemicals (antioxidants, sugars) increase as the fruit ripens, and the maturity stage has a more positive effect on inner quality parameters than stresses such as salinity or frequency of irrigation with saline waters. As fruit ripened from green to red, antioxidant activity and the content of  $\beta$ -carotene, lycopene, total carotenoids, and provitamin A increased. Salinity increased antioxidant activity in the lipophilic fraction and lycopene content [108–110].

Nutrient concentrations that are adequate for crop production under nonsaline conditions may become inadequate and unbalanced under salt stress [15]. Fertilization management becomes important in order to optimize crop yield and quality, particularly when NaCl-dominated irrigation waters are applied. Marin et al. [108] found that pepper fruit quality improved when NaCl-dominated irrigation waters were amended with salts of  $K^+$  and  $Ca^{2+}$  to avoid potentially adverse ion imbalances or interactions. Fruit quality was further enhanced by low irrigation frequency (0.5 events per day) compared to fruit grown under more frequently applied irrigations (8 events per day). In related studies [109,110], increased substrate  $K^+$  in saline irrigation waters promoted vegetative growth and reduced fresh pepper fruit yield. Supplemental  $Ca^{2+}$  had the opposite affect by increasing leaf-Ca, which favored fruit production over vegetative growth and improved the Ca-status of the fruit. Marketable yield increased due to reduction of the number of peppers damaged by BER), a symptom of a physiological disorder generally associated with localized

Ca-deficiency at the fruit tip. Under moderately saline conditions, TSS increased in both green and red fruit with increasing substrate  $K^+$  levels, whereas increases in external-Ca level decreased TSS regardless of fruit ripening stage.

Other investigators [111–113] suggest that low levels of tissue  $Ca^{2+}$  may not be the sole reason for BER. The risk of BER of salt-stressed pepper occurs at the early stages of fruit development, at a time when the levels of ROS peak and antioxidant activity is low in the apoplast of the pericarp. Production of ROS was higher in the more BER-susceptible pepper cultivar than in the BER-resistant cultivar. With fruit development, increases in antioxidant activity were higher in resistant cultivar. Fruit-Ca, however, was not changed by salinity in either cultivar and thus, did not appear to be a major factor in the incidence of BER.

The most important quality trait for paprika sweet pepper is color intensity, a deep-red hue caused by the presence of carotenoids. Fernandez et al. [114] subjected three pepper cultivars to NaCl-salinity levels ranging from 1.1 to 3.6 dS  $m^{-1}$ . Cultivar “Ramillete” was the most salt tolerant with a fruit yield reduction of only 5% at the highest salinity level, compared to yield reductions of 20% and 40% for cultivars “Tres casco” and “Bola,” respectively. Color index of “Ramillete” was highest of the cultivars, increasing 38% as salinity increased.

Tomato (*Lycopersicon esculentum* Mill.). Moderately Sensitive.  $T = 2.5$  dS  $m^{-1}$ ,  $S = 9.9\%$  [11]. Cherry tomato (*L. lycopersicum* var. *cerasiforme* (Dunal) Alef. Moderately sensitive.  $T = 1.7$  dS  $m^{-1}$ ,  $S = 8.1\%$  [11].

Consumption of both fresh and processed tomatoes has increased 40% over the last 20 years, an increase that may be associated with increased consumer interest in the role the bioactive constituents of tomato may play in preventing chronic diseases, improving energy balance, and control of obesity [115]. Tomato consumption is inversely correlated with the risk of certain types of cancer, cardiovascular diseases, and age-related macular degeneration [48]. For fresh fruit consumption, tomato quality is determined by shape, size, color, firmness, texture, absence of blemishes (shoulder cracking, BER), shelf life, aroma, flavor, and nutritional benefits.

The acreage devoted to tomato production has expanded in arid and semiarid regions, and at the same time, fresh waters available for tomato irrigation are dwindling and the quality of available water resources is lower than growers would like. Use of alternative waters (recycled waters, drainage effluents, other poor quality waters), however, presents an environmentally sound system, conserving fresh water, reducing drainage waters, and controlling water pollution problems. Moreover, moderately saline irrigation waters increase quality and consumer acceptability of both large and cherry tomato fruit by improving pigmentation, flavor, sweetness as well as increasing the levels of TSS, sugars, organic acids, and antioxidants including vitamins C and E, lycopene,  $\beta$ -carotene, lutein, and flavonoids [43,50,51,116–123]. Compared to fruit from plants grown under nonsaline conditions, fruit from salt-stressed plants are generally smaller with a thicker and more resistant cuticle and a lower susceptibility to fruit cracking [124], traits that are desirable for processing tomatoes.

Coastal regions in southern Europe are faced with limited sources of irrigation waters and with deterioration of those waters due to seawater intrusion. The response of both round and small-fruited tomato varieties to irrigation with diluted seawater has been addressed by several research teams located in the Mediterranean regions. Irrigation of different tomato genotypes with nutrient solutions amended with 10% seawater ( $EC = 8$  dS  $m^{-1}$ ) [125–127] or 20% seawater ( $EC = 12$  dS  $m^{-1}$ ) [128,129] reduced fruit yield, but not fruit number. All berries were marketable and showed no defects (cuticle cracking) or symptoms of nutritional disorders (BER). Some genotypes showed no increases in phytochemicals in response to salinity, whereas the fruit quality of other salt-stressed cultivars significantly improved through increases in TSS,  $\alpha$ -tocopherol, and ascorbic, lipoic, and dihydrolipoic acids [126–127].

Cherry tomatoes are highly valued by consumers for their superior flavor compared to normal-sized fresh market cultivars [126–129]. Size is critical for cherry tomatoes; optimum diameter is 25–35 mm and fruit deviating from these values have little value in the premium market. Salt-stressed plants produced a higher percentage of fruit of optimal size than those grown under control conditions [130].

Salinity levels that result in improved fruit quality are invariably accompanied by a yield decline. Considerable research has been directed toward developing suitable crop and management practices in order to increase tomato fruit quality without a corresponding tradeoff in yield losses. Cuartero and Fernández-Muñoz [131] give an example: TSS content in ripe processing tomatoes fixes the price paid to the grower. The TSS content in modern tomato hybrids increases at the rate of 10.5% for each additional unit of salinity. In response to irrigation waters equal to or greater than  $2\text{--}2.5\text{ dS m}^{-1}$ , yield of similar hybrids decreases 10% per additional  $\text{dS m}^{-1}$  unit. Thus, the quality  $\times$  quantity index (TSS and ton per ha) remains unchanged at least between 2.5 and  $8\text{--}9\text{ dS m}^{-1}$ . Stephanie DePascale and colleagues [120] also reported that, with appropriate management, an economically viable balance between tomato yield and quality could be achieved under field conditions. Provided the EC of the NaCl-dominated irrigation waters did not exceed  $4.4\text{ dS m}^{-1}$ , marketable fruit yield was not reduced and yet fruit quality improved significantly through increases in TSS, lycopene, and total carotenoids. In contrast, Krauss et al. [46] reached a completely different conclusion based on a comprehensive overview of growth, yield, and fruit quality characteristics of a salad tomato hybrid subjected to three levels of NaCl-dominated salinity ( $\text{EC}_{\text{iw}} = 3, 6.5$  and  $10\text{ dS m}^{-1}$ ) in greenhouse soil-less cultures. Total fruit yield was not reduced by moderate salinity, but decreased 40% at the highest salinity level. Fruit quality, as measured by TSS, vitamin C,  $\beta$ -carotene, lycopene, phenolics, and citric acid, improved only when the EC of the irrigation waters reached  $10\text{ dS m}^{-1}$ . These and other investigators [132] concluded that the substantial yield losses due to salinity were unlikely to be balanced by higher prices for a healthier product, even when the reduced costs of irrigating with degraded water are factored into the calculations. Kan [133], however, presented an economic analysis showing that the positive impact of irrigation with saline water on quality of processing tomatoes in California exceeds its negative impact on crop yield. The economic model considers optimal water management under environmental regulations associated with drainage effluent disposal in California.

Timing of salt stress application to crops can alter yield, yield components, and quality parameters. The duration of stress periods may be very brief. Adams and Ho [134–136] compared the consequences of constant salinity ( $8\text{ dS m}^{-1}$ ) and salinity fluctuating from  $8\text{ dS m}^{-1}$  during the day to  $3\text{ dS m}^{-1}$  during the night. The authors concluded that fluctuating salinity had no advantage over the use of constant salinity on tomato yield and quality. In a similar study, Santamaria et al. [137] found that nutrient solutions with high  $\text{EC}_{\text{iw}}$  ( $6\text{ dS m}^{-1}$ ) applied during the night and the same nutrient solutions with low  $\text{EC}_{\text{iw}}$  ( $2\text{ dS m}^{-1}$ ) applied during the day improved tomato quality without affecting fruit yield.

Even a relatively short delay in salinization may ameliorate tomato yield losses. Application of salinity ( $\text{EC}_{\text{iw}} = 7.5\text{ dS m}^{-1}$ ) starting at the fourth-leaf stage reduced the yield of processing tomatoes 30% compared to the 60% loss measured under continuous salinity from planting [138]. Mizrahi [139] reported that moderate NaCl-salinity applied to hydroponically grown tomato at flowering increased sugars, acids, pigments, and flavor, but at the same time decreased yield 10%–20%, depending on the cultivar. The effect of salt stress initiated at even later growth stages showed great promise for maximizing fruit quality without significant yield loss [140,141]. TSS, lycopene, fructose, and glucose concentrations were enhanced in salad tomatoes if salinization was applied immediately after anthesis or further delayed for 4 weeks after anthesis [141]. Salinity applied at the immature green stage (2 weeks after flowering) increased TSS, titratable acidity, and ascorbic acid compared to the nonsaline controls, but decreased fruit yield 50%. When salinization was delayed until the decoloring stage (6 weeks after flowering), TSS and titratable acidity increased, ascorbic acid was unaffected, and fruit yield was not significantly different from the controls [142].

Several research teams point out that the negative effects of salinity on tomato yield can be alleviated if the external solution is balanced and nutritionally adequate [130,132,143–145]. The N source in saline irrigation waters may affect yield, quality, and post-harvest handling. Moderate salinity (30 mM NaCl) throughout the production cycle did not reduce yield when  $\text{NO}_3^-$  was the only N source, but caused only modest increases in fruit quality. Salinity and  $\text{NH}_4^+$  increased the content



of sugars and organic acids, but decreased yields [145]. Ben-Oliel et al. [145] found that  $\text{NO}_3^-/\text{NH}_4^+$  ratios of 7:1 improved fruit size without significant loss of fruit quality. External  $\text{Cl}^-$  in the salinizing media tended to reduce  $\text{NO}_3^-$  uptake and accumulation [48] and, as a result, the  $\text{NO}_3^-$  content of salinized tomatoes was reduced, a highly desirable trait for the canning industry. High  $\text{NO}_3^-$  may cause spoilage through detinning of the internal can surface [120].

Quality of tomato fruit is often adversely affected by BER even under nonsaline conditions [48], but the incidence of BER increases when the crop is under salt stress [131,134,135]. The symptoms may begin very early. Inflorescences fail to develop normally [146,147], the distal placental tissue degrades, the pericarp becomes necrotic, and the fruit is unmarketable [131,147–150]. Numerous investigators agree that the disorder is not caused by the presence of a toxic ion, but rather by the absence of an essential mineral nutrient [48]. Salinity-induced increases in the incidence of BER appear to be caused by  $\text{Ca}^{2+}$  deficiency due to ion interactions in the external substrate that decrease  $\text{Ca}^{2+}$  availability to the plant [146], to increased resistance to  $\text{Ca}^{2+}$  uptake and transport to the shoot, and to inefficient partitioning of  $\text{Ca}^{2+}$  to reproductive organs [48].

Grafting techniques to increase the productivity of tomato growing under salt stress depend on the unique salt tolerance mechanisms that both the scion and rootstock contribute to the grafted plant. Whether the rootstock or the scion regulates toxic ion transport is controversial. One prevailing opinion is that the rootstock genotype plays the major role in the success of the grafted plant [151,152]; another viewpoint is that the scion genotype controls ion uptake and plant growth [153,154]. Grafting has little effect on quality of fruit grown under salt stress. TSS, glucose, and fructose in both grafted and ungrafted plants increased with salinity. In one study, salinity had no effect on  $\beta$ -carotene or lycopene, whereas the effect of grafting was strong for both constituents. Fruit on grafted plants growing under nonsaline conditions, for example, had twice the lycopene content as those on ungrafted control plants [53].

## 47.5 SALINITY EFFECTS ON QUALITY OF OIL-PRODUCING CROPS

Olive (*Olea europaea* L.). Moderately salt tolerant [11]. Based on a nine-year study conducted in the Negev Desert of Israel, Wiesman et al. [155] concluded that olive production was sustainable under irrigation with moderately saline local waters ( $\text{EC} = 4.2 \text{ dS m}^{-1}$ ). Saline treatments significantly improved the oil content of polyphenols and vitamin E and tended to increase the oil yield and oil percentage. The effect of salinity on these yield components was not always significant [155,156]. From a two-year field trial conducted at Sfax, Tunisia, Ben Ahmed et al. [157] reported that saline irrigation ( $\text{EC}_{\text{iw}} = 7.5 \text{ dS m}^{-1}$ ) decreased olive fruit yield 42% and total oil content 8% compared to production from trees irrigated with  $2 \text{ dS m}^{-1}$  waters. Oil quality, however, was enhanced by salinity due to significant increases in major phenolic constituents and by higher concentrations of oleic, linoleic, and linolenic acids.

Coriander (*Coriandrum sativum* L.). No available information on salt tolerance of this crop. Fresh leaves of coriander, a valuable culinary herb commonly known as cilantro or Chinese parsley, have a unique aroma due to a complex mixture of essential oils. Oil yield from leaves of plants irrigated during the vegetative stage with saline waters (50 mM NaCl) were 43% higher than from leaves of plants irrigated with nonsaline water, a response probably due to increases in oil gland density and the absolute number of glands. Oil composition was also affected by salinity; constituents such as dodecanal, aldehydes, and monoterpenic alcohols increased compared to the control [158].

Lesquerella (*Lesquerella fendleri* (Gray) S. Wats.). Tolerant.  $T = 6.1 \text{ dS m}^{-1}$ ,  $S = 19\%$  [159]. Lesquerella (desert mustard) is a promising new crop with seed oil containing lesquerolic acid, a hydroxy fatty acid used in the production of lubricants, plastics, and cosmetics. Results from a trial conducted in sand cultures irrigated with waters prepared to simulate the saline drainage effluents commonly present in the San Joaquin Valley of California showed that both seed yield and extractable seed oil content increased as salinity rose from 3 to  $11 \text{ dS m}^{-1}$ . Lesquerolic acid content was also significantly higher in saline treatments above  $11 \text{ dS m}^{-1}$  compared to the control [160,161].

Evening primrose (*Oenothera biennis* L.). No available information on salt tolerance of this crop. Evening primrose is a rich source of polyunsaturated fatty acids that are linked to lowering blood cholesterol levels and reducing the risk of atherosclerosis. As salinity rose from 1 to 3 dS m<sup>-1</sup>, seed oil content increased, and the content of palmitic, stearic, and  $\gamma$ -linolenic acids rose. The favorable ratio of omega-6 to omega-3 fatty acids in the oil was also a positive effect of salinity inasmuch as the ratio in human diets plays an essential role in control of membrane polyenoic fatty acids [162].

Stock (*Matthiola incana* (L.) R. Br.). Tolerant.  $T = 8 \text{ dS m}^{-1}$ ,  $S = 3\%$  [163]. Stock is not only a commercially important cut flower and bedding plant, but is also a valuable oilseed crop. The oils are rich in omega-3 linolenic acid, a fatty acid that has been linked to a variety of potentially therapeutic effects contributing to reduction in cardiovascular diseases in humans [164]. Seed oil content of greenhouse stock plants remained constant in response to salinity treatments ranging from 1 to 8 dS m<sup>-1</sup>; seed yield was not significantly reduced until salinity exceeded 4 dS m<sup>-1</sup>. The percentage of linolenic acid in the oil increased significantly from 50% to 54% as salinity increased [165].

## 47.6 SALINITY EFFECTS ON QUALITY OF MEDICINAL CROPS

Interest in the use of medicinal plant products has proceeded apace over the past few years, as evidenced by their wide availability in retail outlets and by the increased attention they are receiving in public media. The medicinal effects for humans undoubtedly result from the combined effects of secondary metabolites that the plant biosynthesizes to defend against both biotic (herbivory, pathogen attack, interplant competition) and abiotic (water and nutrient status, changes in temperature, light levels, salinity) stresses. Research in the pharmacognosy of medicinal plants has focused on assays of bioactivity, identification of potential modes of action, and target sites for active phytochemical compounds [166,167].

*Echinacea* species. No available salt tolerance information. Coneflowers (*Echinacea* species) are herbaceous perennials native to North America. The expressed juice of the aerial portion of *E. purpurea* and alcohol extracts of *E. angustifolia* and *E. pallida* roots are widely used as nonspecific stimulants for the immune system [167,168] and are said to be beneficial in reducing the symptoms and perhaps the duration of upper-respiratory infections [167]. In response to low salinity (NaCl = 50 mM), the content of chlorogenic acid, cynarin and cichoric acid, and total phenolics in roots of *E. angustifolia* grown in hydroponic cultures significantly increased [166], as did the content of caftaric and cynarin in *E. purpurea* leaves [169]. When NO<sub>3</sub><sup>-</sup> was the sole source of N in the saline solutions, concentrations of chlorogenic acid, echinacoside, caffeic acid, and total phenolics increased in root tissues. The authors suggest that improved nutrient and salinity management practices may result in increased concentrations of the active constituents in this crop [169].

*Digitalis purpurea* L. No available salt tolerance information. Purple foxglove, a member of the plant family Plantaginaceae (formerly Scrophulariaceae), produces cardenolides, the aglycone constituents of cardiac glycosides, in flowers, seeds, and leaves. The main cardenolides, digitoxin and digoxin, are used as a medication for heart failure. The cardenolide content in leaves of 42 days old *D. purpurea* plants irrigated with moderately saline waters (100 mM NaCl) was double that in leaves of the control plants [170].

## 47.7 SALINITY EFFECTS ON QUALITY OF FLORICULTURAL CROPS

Numerous cut flower species are moderately tolerant to salinity, as determined by stem length, flower size, and number. Statice (*Limonium perezii* (Stapf.) F. T. Hubb.), *L. sinuatum* (L.) Mill., *L. latifolium* (Sm.) O. Kuntz), stock (*Matthiola incana* (L.) R. Br.), snapdragon (*Antirrhinum majus* L.), sunflower (*Helianthus annuus* L.), and carnation (*Dianthus chinensis* L.) produce premium, marketable flowering stems at soil salinity values (ECe) as high as 5 dS m<sup>-1</sup> [171]. Chrysanthemum (*Chrysanthemum morifolium* Ramat) and celosia (*Celosia argenta* L.) are equally salt tolerant, but for these crops that tend to be excessively tall and rangy under nonsaline conditions, height control is an important

quality issue. With proper management, salinity offers a substantial benefit: an environmentally friendly alternative to chemical growth retardants to restrict height and strengthen stems [172,173].

Important quality characteristics for landscape plants are aesthetic value, sustainability, and growth habit. Under the appropriate level of salinity stress, selected plants are good candidates for salt-affected sites; these and many other species are slow-growing and stunted, but without overt symptoms of ion toxicity or deficiency [174–176]. Control of height and branching by salinity is a benefit due to reduction in maintenance costs.

After some optimum level of vegetative growth has been reached under nonsaline conditions, application of salinity to cut flower crops tends to enhance reproductive growth by increasing the number of flowers. Shillo et al. [177] reported that irrigating lisianthus (*Eustoma grandiflorum* L.) from seedling transplant to flower bud appearance with nonsaline waters, and thereafter applying saline waters ( $EC_{iw} = 6 \text{ dS m}^{-1}$ ) to harvest, increased quality of the flowering stems. Stem weight and diameter as well as the number of flowers per stem increased in response to applied salinity; stem and inflorescence lengths were not affected by saline treatment.

## 47.8 SUMMARY

With advice from the USDA food guide pyramid program [178], the American public has become more health conscious, more knowledgeable about the importance of a healthy diet. The general dietary advice suggests that we increase our intake of fruits and vegetables. Epidemiological studies indicate that diets high in fruits and vegetables contribute to the prevention of chronic and age-related degenerative diseases as well as to weight control, particularly to the control of juvenile obesity. The bioactive constituents responsible for the health benefits in plant-based foods also play a significant role in consumer perception of specific quality attributes: flavor, taste, color, aroma, visual appeal, and nutrition.

Biosynthesis of many of these phytochemicals is stimulated by abiotic environmental stresses, including salinity. Both inner (phytochemical content) and outer (firmness, texture, appearance) crop quality characteristics improve with salt stress. The past two decades have witnessed renewed interest in improvement in crop quality through the controlled use of saline irrigation waters. Innovative procedures for balancing increases in quality against salinity-induced yield reductions have been developed. At the same time, water management practices have been improved to the point that diluted seawater, agricultural drainage effluents, greenhouse run-off waters, and recycled and other low-quality waters are routinely used successfully for irrigated agriculture, thereby conserving potable waters for domestic use. In combination, improvements of environmental stewardship together with improvements in the management of saline waters for the production of high-value, high-quality crops offer multiple benefits to both producers and consumers.

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